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(54) Title: MESOTHELIN VACCINES AND MODEL SYSTEMS

(57) Abstract: Mesothelin can be used as an immunotherapeutic target. It induces a cytolytic T cell response. Portions of mesothelin which induce such responses are identified. Vaccines can be either polynucleotide- or polypeptide-based. Carriers for raising a cytolytic T cell response include bacteria and viruses. A mouse model for testing vaccines and other anti-tumor therapeutics and prophylactics comprises a strongly mesothelin-expressing, transformed peritoneal cell line.

MESOTHELIN VACCINES AND MODEL SYSTEMS

[01]   The contents of each of the following applications are specifically incorporated herein: provisional U.S. Applications Ser. No. 60/395,556, filed July 12, 2002, 60/398,217, filed July 24, 2002, Ser. No. 60/414,931, filed September 30, 2002, and Ser. No. 60/475,783 filed June 5, 2003.

FIELD OF THE INVENTION

[04]   The invention relates to the field of cancer therapeutics, cancer prognosis, and anti-cancer drug development. In some aspects it relates to mesothelin as a therapeutic target. In another aspect it relates to developing other therapeutic targets.

BACKGROUND OF THE INVENTION

[05]   Transformation from a normal to a malignant cell involves complex genetic and epigenetic changes, affecting a large number of genes (1, 2). Many of these altered genes are translated into new, altered, or overexpressed proteins that may represent candidate targets for immune rejection. T cell screening of cDNA libraries isolated from tumor cells, biochemical elution and purification of major histocompatibility complex (MHC) bound antigens, and antibody screening of phage display libraries (SEREX method) have greatly facilitated the identification of tumor antigens, particularly those expressed by malignant melanomas (3-13). As a result, there are a number of antigen-specific vaccine approaches under clinical development for this disease (3-6, 14). Unfortunately, these antigen identification approaches have not been successful for

1

identifying antigens expressed by many other common cancers. The major limitation has been the inability to generate patient-derived T cell lines and clones that can be employed to identify immune relevant tumor targets. Furthermore, T cell responses to specific human tumor antigens have not yet been correlated with clinical responses after immunotherapy.

[06]   The recent development of high throughput technologies that can quantify gene expression in human tissues has led to the identification of a large number of genes that are differentially expressed in tumors relative to the normal tissue from which they derive (15-18). These gene expression databases can be used as initial filters upon which to apply a functional immune-based screening strategy (19). A growing number of genes shown to be differentially expressed in pancreatic adenocarcinomas using serial analysis of gene expression (SAGE) have been tabulated and reported (20-22). However, it is unclear which of these differentially expressed genes are immunologically relevant for an anti-tumor response. There is a need in the art for a way of identifying immunologically relevant proteins among the proteins which are differentially expressed in tumor and normal tissues.

BRIEF SUMMARY OF THE INVENTION

[07]   In a first embodiment a method is provided for inducing a T-cell response to a tumor that overexpresses mesothelin relative to normal tissue from which the tumor is derived. The tumor can be, for example, an ovarian cancer, a pancreatic cancer, a mesothelioma, or a squamous cell carcinoma. A vaccine comprising a polypeptide comprising an MHC Class I- or Class II-binding epitope of mesothelin is administered to a patient who has said tumor or who has had said tumor removed. The patient can also be one who is at risk of developing such a tumor. The epitope binds to an allelic form of MHC class I or MHC class II which is expressed by the patient. A T-cell response to mesothelin is thereby induced. The vaccine does not comprise whole tumor cells. The polypeptide is optionally mesothelin. The T-cell response may be a CD4$^+$ T-cell response and/or a CD8$^+$ T-cell response.

[08]   In a second embodiment a method is provided for inducing a T-cell response to a tumor that overexpresses mesothelin relative to normal tissue from which the tumor is derived. The tumor can be, for example, an ovarian cancer, a pancreatic cancer, a mesothelioma, or a squamous cell carcinoma. A vaccine comprising a polynucleotide encoding a

polypeptide comprising an MHC Class I- or MHC Class II-binding epitope of mesothelin is administered to a patient who has said tumor or who has had said tumor removed. The patient can also be one who is at risk of developing such a tumor. The epitope binds to an allelic form of MHC class I or class II which is expressed by the patient. A T-cell response to mesothelin is thereby induced. The vaccine does not comprise whole tumor cells. The polypeptide encoded by the polynucleotide of the vaccine is optionally mesothelin. The T-cell response may be a $CD4^+$ T-cell response and/or a $CD8^+$ T-cell response.

[09] In a third embodiment a method is provided for identifying immunogens useful as candidates for anti-tumor vaccines. A protein is selected which is expressed by a tumor and which is minimally or not expressed by normal tissue from which the tumor is derived. Preferably the protein is expressed by a greater than 10 % of tumor isolates tested of a type of tumor. Lymphocytes of humans who have been vaccinated with a vaccine which expresses the protein are tested to determine if the lymphocytes comprise $CD8^+$ T cells or $CD4^+$ T cells which are specific for the protein. The presence of the $CD8^+$ T cells or $CD4^+$ T cells indicates that the protein is a candidate for use as an anti-tumor vaccine.

[10] A fourth embodiment of the invention provides a method of predicting future response to a tumor vaccine in a patient who has received the tumor vaccine. Lymphocytes of the patient are tested to determine if the lymphocytes comprise $CD8^+$ T cells or $CD4^+$ T cells which are specific for an antigen in the vaccine. The presence of said $CD8^+$ T cells or $CD4^+$ T cells predicts a longer survival time than the absence of said $CD8^+$ T cells or $CD4^+$ T cells.

[11] A fifth embodiment of the invention provides a vaccine which induces a $CD8^+$ T cell or $CD4^+$ T cell response. The vaccine comprises a polypeptide comprising an MHC Class I- or MHC Class II-binding epitope of mesothelin. The epitope binds to an allelic form of MHC class I or class II which is expressed by the patient. A T-cell response to mesothelin is thereby induced. The vaccine does not comprise whole tumor cells. The vaccine further comprises a carrier for stimulating a T cell immune response. The polypeptide is optionally mesothelin.

[12] Another embodiment of the invention provides another vaccine which induces a $CD8^+$ T cell or $CD4^+$ T cell response. The vaccine comprises a polynucleotide encoding a

polypeptide comprising an MHC Class I- or MHC Class II-binding epitope of mesothelin. The epitope binds to an allelic form of MHC class I or class II which is expressed by the patient. A $CD8^+$ T cell or $CD4^+$ T cell response to mesothelin is thereby induced. The vaccine does not comprise whole tumor cells. The vaccine further comprises a carrier for stimulating a T cell immune response. The polypeptide encoded by the polynucleotide of the vaccine is optionally mesothelin.

[13]   Another embodiment of the invention provides an isolated polypeptide of 9 to 25 amino acid residues. The polypeptide comprises an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

[14]   Yet another embodiment of the invention provides an antibody that binds to an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

[15]   Yet another embodiment of the invention provides a $CD8^+$ T cell or $CD4^+$ T cell line that binds to MHC class I-peptide complexes, wherein the peptide comprises an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

[16]   A tenth embodiment of the invention provides a method for predicting future response to a tumor vaccine in a patient who has received the vaccine. The tumor vaccine comprises at least one T-cell epitope of mesothelin. The patient is tested to determine if the patient has a delayed type hypersensitivity (DTH) response to mesothelin, wherein the presence of said response predicts a longer survival time than the absence of said response.

[17]   An eleventh embodiment of the invention provides a recombinant mouse cell line which comprises peritoneal cells which have been transformed by HPV-16 genes E6 and E7 and an activated oncogene. The cell line is capable of forming ascites and tumors upon intraperitoneal injection into an immunocompetent mouse.

4

[18] Also provided is a mouse model which comprises a mouse which has been injected with a recombinant mouse cell line. The recombinant mouse cell line comprises peritoneal cells transfected by HPV-16 genes E6 and E7 and an activated oncogene. The former genes immortalize and the latter gene transforms. The cell line is capable of forming ascites and tumors upon intraperitoneal injection into an immunocompetent mouse.

[19] Another aspect of the invention is a method of testing a substance to determine if it is a potential drug for treating a cancer. The cancer may be, for example, an ovarian cancer, a pancreatic cancer, a mesothelioma, or a squamous cell carcinoma. A test substance is contacted with a mouse model. The mouse model comprises a mouse that has been injected with a recombinant mouse cell line. The injection can be accomplished before or after the test substance is contacted with the mouse. The recombinant mouse cell line comprises peritoneal cells which have been transfected by HPV-16 genes E6 and E7 and an activated oncogene. The cell line is capable of forming ascites and tumors upon intraperitoneal injection into an immunocompetent mouse. One determines whether the test substance causes delay of tumor formation or regression of a tumor in the mouse model, diminution of ascites volume in the mouse model, or longer survival time in the mouse model. Any of these effects indicates that the test substance is a potential drug for treating cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[20] Figs. 1A to 1F show a T2 binding assay that identifies mesothelin and PSCA protein derived epitopes that bind to HLA-A2, A3, and A24 molecules. T2 cells were pulsed with 100-400 micrograms of peptide overnight at room temperature before analysis by flow cytometry. Fig. 1A. T2 cells expressing HLA-A2 and pulsed with either: no peptide (black line), a Mesothelin A1309-318 binding peptide (green line), Mesothelin A220-29 (pink line), and Mesothelin A2530-539 (blue line). Peptide pulsed cells were stained with an unlabeled mouse anti-HLA class I molecule monoclonal antibody W6/32 and a goat-anti-mouse FITC-labeled IgG2a secondary antibody. Fig. 1B. T2 cells genetically modified to express A3 and pulsed with either: no peptide (black line), Mesothelin A1309-318 binding peptide (green line), Mesothelin A383-92 (pink line), and Mesothelin A3225-234 (blue line). Peptide pulsed cells were stained with an unlabeled mouse anti-human HLA-A3 specific monoclonal antibody GAPA3 and a FITC-labeled IgG2a secondary antibody. Fig. 1C. T2 cells genetically modified to express A24 and

pulsed with either: no peptide (black line), Mesothelin A1309-318 peptide (green line), Mesothelin A24435-444 (pink line), and Mesothelin A24475-484 (blue line). Peptide pulsed cells were stained with an unlabeled pan-HLA antibody W6/32 and a FITC-labeled IgG2a secondary antibody. Fig. 1D. T2 cells expressing HLA-A2 and pulsed with either: Mesothelin A1309-318 binding peptide (green line), PSCA A25-13 (pink line), PSCA A214-22 (blue line), PSCA A2108-116 (orange line)and PSCA A243-51 (red line). Peptide pulsed cells were stained with an unlabeled mouse anti-HLA class I molecule monoclonal antibody W6/32 and a goat-anti-mouse FITC-labeled IgG2a secondary antibody. Fig. 1E.· T2 cells genetically modified to express A3 and pulsed with either: Mesothelin A1309-318 binding peptide (green line), PSCA A399-107 (pink line), A35-13 (blue line), A314-22 (orange line), A3109-117 (purple line), A343-51 (red line), and PSCA A320-28 (yellow line). Peptide pulsed cells were stained with an unlabeled mouse anti-human HLA-A3 specific monoclonal antibody GAPA3 and a FITC-labeled IgG2a secondary antibody. Fig. 1F. T2 cells genetically modified to express A24 and pulsed with either: Mesothelin A1309-318 peptide (green line), PSCA A2476-84 (pink line), PSCA A24108-116 (blue line), PSCA A2499-107 (orange line), PSCA A24109-117 (purple line), and PSCA A2477-85 (red line). Peptide pulsed cells were stained with an unlabeled pan-HLA antibody W6/32 and a FITC-labeled IgG2a secondary antibody.

[21]   Figs. 2A to 2D shows an ELISPOT analysis of CD8+ T cells from PBMCs which demonstrates post-vaccination induction of mesothelin-specific T cells in three DTH responders but not in 11 non-DTH responders who received an allogeneic GM-CSF-secreting tumor vaccine for pancreatic cancer. Fig. 2A. ELISPOT analysis of PBL from two patients who were HLA-A3 positive; Fig. 2B. ELISPOT analysis of PBL from two patients who were HLA-A 2 and HLA-A3 positive; Fig. 2C. ELISPOT analysis of PBL from two patients who were HLA-A24 positive. Fig. 2D. ELISPOT analysis was performed on PBL from all 14 patients who were treated on the phase I allogeneic GM-CSF secreting pancreatic tumor vaccine study (28). ELISPOT analysis for IFN-γ-expressing cells was performed using PBMC that were isolated on the day prior to vaccination or 28 days following the first vaccination. Lymphocytes were isolated by ficoll-hypaque separation and stored frozen in liquid nitrogen until the day of assay. CD8+ T cell enrichment was performed prior to analysis. T2-A3 cells were pulsed with the two mesothelin derived epitopes MesoA3(83-92) (open squares), MesoA3(225-234)

(closed circle) and HIV-NEFA3 (94-103) (open triangle). T2-A2 cells were pulsed with the two mesothelin derived epitopes MesoA2(20-29) (closed squares), MesoA2(530-539) (open circle), and HIV-GAG(77-85), (closed triangle). T2-A24 cells were pulsed with the two mesothelin derived epitopes MesoA24 (435-444) (open diamond), MesoA24(475-484) (closed diamond), and tyrosinase A24(206-214) (star). All DTH responders are represented by red lines, and DTH non-responders are represented by black lines. For the detection of nonspecific background, the number of IFN-γ spots for CD8+ T cells specific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-GAG protein derived epitope (SLYNTVATL; SEQ ID NO:7), the HLA-A3 binding HIV-NEF protein derived epitope (QVPLRPMTYK; SEQ ID NO: 8), and the HLA-A24 binding tyrosinase protein derived epitope (AFLPWHRLF; SEQ ID NO: 9) were used as negative control peptides in these assays. Data represents the average of each condition assayed in triplicate and standard deviations were less than 5%. Plotted are the # of human interferon gamma (hIFNg) spots per 105 CD8+ T cells. Analysis of each patient's PBL was performed at least twice.

[22] Fig. 3 shows an ELISPOT analysis performed to assess the recognition of the influenza matrix protein HLA-A2 binding epitope M1 (GILGFVFTL; SEQ ID NO: 10) on PBL from all 5 patients on the study who were HLA-A2 positive (4 non-DTH responders and 1 DTH responder). This analysis was performed on the same PBL samples described for Figs. 2 A to 2D above. The DTH responders are represented by red lines, and the DTH non-responders are represented by black lines. For the detection of nonspecific background, the number of IFN-γ spots for CD8+ T cells specific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-GAG protein derived epitope (SLYNTVATL; SEQ ID NO: 7), the HLA-A3 binding HIV-NEF protein derived epitope (QVPLRPMTYK; SEQ ID NO: 8), and the HLA-A24 binding melanoma tyrosinase protein derived epitope (AFLPWHRLF; SEQ ID NO: 9) were used as negative control peptides in these assays. Data represents the average of each condition assayed in triplicate and standard deviations were less than 5%. Plotted are the # of human interferon gamma (hIFNg) spots per 105 CD8+ T cells. Analysis of each patient's PBL was performed at least twice and all ELISPOT assays were performed in a blinded fashion.

[23] Fig. 4A to 4D shows an ELISPOT analysis of CD8+ T cells from PBMCs. No post-vaccination induction was observed of PSCA-specific T cells in DTH responders or non-

DTH responders who received an allogeneic GM-CSF-secreting tumor vaccine for pancreatic cancer. Fig. 4A. ELISPOT analysis of PBL from two patients who were HLA-A3 positive; Fig. 4B. ELISPOT analysis of PBL from two patients who were HLA-A 2 and HLA-A3 positive; Fig. 4C. ELISPOT analysis of PBL from two patients who were HLA-A24 positive. Fig. 4D. ELISPOT analysis of PBL from eight patients who were non-responders. ELISPOT analysis for IFN-γ-expressing cells was performed using PBMC that were isolated on the day prior to vaccination or 28 days following each of the vaccination. Lymphocytes were isolated by ficoll-hypaque separation and stored frozen in liquid nitrogen until the day of assay. CD8+ T cell enrichment was performed prior to analysis. T2-A3 cells were pulsed with the six PSCA derived epitopes: PSCAA3(7-15) (closed squares), PSCAA3(52-60) (closed diamond), PSCAA3(109-117) (closed triangle), PSCAA3(43-51) (open square), PSCAA3(20-28) (open diamond), and PSCAA3(99-107) (open triangle). Negative HIV-NEFA3 (94-103) values were subtracted out. T2-A2 cells were pulsed with the three PSCA derived epitopes: PSCAA2(5-13) (closed squares), PSCAA2(14-22) (closed diamonds), PSCAA2(108-116) (closed triangles). Negative HIV-GAG(77-85) values were subtracted out. T2-A24 cells were pulsed with the five PSCA derived epitopes: PSCAA24(76-84) (closed diamond), PSCAA24(77-85) (star), PSCAA24(109-117) (closed triangles), PSCAA24(108-116) (closed circle), and PSCAA24(99-107) (open triangle). Negative Tyrosinase A24(206-214) values were subtracted. All DTH responders are represented by red lines, and DTH non-responders are represented by black lines. For the detection of nonspecific background, the number of IFN-γ spots for CD8+ T cells specific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-GAG protein derived epitope (SLYNTVATL; SEQ ID NO: 7), the HLA-A3 binding HIV-NEF protein derived epitope (QVPLRPMTYK; SEQ ID NO: 8), and the HLA-A24 binding tyrosinase protein derived epitope (AFLPWHRLF; SEQ ID NO: 9) were used as negative control peptides in these assays. Data represents the average of each condition assayed in triplicate and standard deviations were less than 5%. The number of human interferon gamma (hIFNg) spots per 105 CD8+ T cells is plotted. Analysis of each patient's PBL was performed at least twice.

[24]   Fig. 5 shows expression of surface Mesothelin and PSCA on Panc 6.03 and Panc 10.05 vaccine lines. The pancreatic tumor vaccine lines Panc 6.03 (top two panels) and Panc 10.05 (bottom two panels) were analyzed by flow cytometry for their levels of surface

mesothelin and PSCA using the mesothelin specific monoclonal antibody CAK1 (left panels) and the PSCA specific monoclonal antibody 1G8 (right panels) as the primary antibody and goat anti-mouse IgG FITC as the secondary antibody. The solid line represents the isotype control, the green shaded area represents mesothelin staining, and the pink shaded area PSCA staining.

[25]   Figs. 6A to 6C show that mesothelin-specific CD8+ T cells are detected following multiple vaccinations with an allogeneic GM-CSF secreting tumor vaccine in DTH-responders but not in non-DTH responders. Fig. 6A. ELISPOT analysis of PBL from two patients who were HLA-A3 positive; Fig. 6B. ELISPOT analysis of PBL from two patients who were HLA-A 2 and HLA-A3 positive; Fig. 6C. ELISPOT analysis of PBL from two patients who were HLA-A24 positive. ELISPOT analysis for IFN-γ-expressing cells was performed using PBMC that were isolated on the day prior to vaccination or 28 days following each vaccination as described in Fig. 2A to 2D. Each peptide has the same symbol code as described for Fig. 2A to 2D. The DTH responders are represented by the red lines and the DTH non-responders are represented by the black lines. For the detection of nonspecific background, the number of IFN-γ spots for CD8+ T cells specific for the irrelevant control peptides were counted. The HLA-A2 binding HIV-GAG protein derived epitope (SLYNTVATL; SEQ ID NO: 7), the HLA-A3 binding HIV-NEF protein derived epitope (QVPLRPMTYK; SEQ ID NO: 8), and the HLA-A24 binding melanoma tyrosinase protein derived epitope (AFLPWHRLF; SEQ ID NO: 9) were used as negative control peptides in these assays. Data represent the average of each condition assayed in triplicate and standard deviations were less than 5%. Plotted are the number of human interferon gamma (hIFNg) spots per 105 CD8+ T cells. Analysis of each patient's PBL was performed at least twice.

[26]   Figs. 7A to 7C show the generation and characterization of an ascitogenic ovarian tumor cell line (WF-3) in mice. WF-3 tumor cells were injected into C57BL/6 mice intraperitoneally at a dose of 1×10^5 cells/mouse. Mice were euthanized 4 weeks after tumor challenge (7A) Representative gross picture to demonstrate ascites formation in mice. Note: Mice developed significant ascites with an increase in abdominal girth 4 weeks after tumor challenge. (7B) Hematoxylin and eosin staining of the explanted tumors viewed at 90x magnification. The tumors displayed a papillary configuration, morphologically consistent with tumors derived from the peritoneum or ovaries. Tumors

viewed at 400x magnification. The inset displays the features of a WF-3 tumor cell in greater detail.

[27]   Figures 8A and 8B show MHC class I (Figure 8A) and MHC class II (Figure 8B) presentation on the mouse WF-3 tumor cells.   WF-3 tumor cells were harvested, trypsinized, washed, and resuspended in FACSCAN buffer. Anti-H2Kb/H-2D monoclonal antibody or anti-I-Ab monoclonal antibody was added, followed by flow cytometry analysis to detect MHC class I and class II expression on WF-3 tumor cells. (8A) WF-3 tumor cells were positive for MHC class I presentation (thick line) compared to the MHC class I-negative control (thin line). (8B) WF-3 tumor cells were negative for MHC class II presentation. The thin line indicates staining of the MHC class II-negative control.

[28]   Figures 9A to 9B show the effect of WF-3 tumor dose on ascites formation in two independent trials shown in Figure 9A and Figure 3B. WF-3 tumor cells were injected into C57BL/6 mice intraperitoneally at various doses (1×10^4 , 5×10^4, 1×10^5 , and 1×10^6 cells/mouse). Mice were monitored twice a week for ascites formation and tumor growth. Note: All of the mice injected with 5×10^4, 1×10^5, and 1×10^6 cells intraperitoneally, developed ascites and tumor growth within 30 days. 20% of mice injected with 1×10^4 cells were tumor-free without ascites formation after 90 days of tumor injection. The data are from one representative experiment of two performed.

[29]   Fig. 10 shows expression of murine mesothelin in WF-3 tumor cells demonstrated by RT-PCR with gel electrophoresis. Fig. 10. RT-PCR. RT-PCR was performed using the Superscript One-Step.RT-PCR Kit (Gibco, BRL) and a set of primers: 5'-CCCGAATTCATGOCCTTGCCAACAGCTCGA-3' (SEQ ID NO: 11) and 5'-TATGAATCCGCTCAGCCTTAAAGCTGGGAG-3' (SEQ ID NO: 12). Lane 1, size marker. Lane 2, RNA from W-3 cells and Lane 3, RNA from mesothelin-negative B 16 tumor cells. Specific amplification (indicated by an arrow) was observed in Lane 2 (WF-3 cells) but not in the Lane 3 (B16 cells).

[30]   Figure 11 shows in vivo tumor protection experiments against WF-3 tumor growth using mesothelin-specific DNA vaccines. Mice received a booster with the same dose one week later, followed by intraperitoneal challenge with 5×10^4 WF-3 cells/mouse one week afterward. Ascites, formation in mice was monitored by palpation and inspection. Mice were, sacrificed at day 90. Note: Vaccination with pcDNA3-mesothelin DNA

resulted in a significantly higher percentage of tumor-free mice than vaccination with other DNA. (P<0.00 1). Results shown here are from one representative experiment of two performed.

[31]   Figure 12 shows CTL assays which demonstrate specific lysis induced by vaccination with mesothelin-specific DNA vaccines. Mice (5 per group) were imunized with various DNA vaccines intradermally. Mice received a booster with the same dose one week later. Splenocytes from mice were pooled 14 days after vaccination. To perform the cytotoxicity assay, splenocytes were cultured with mesothelin protein-for 6 days and used as effector cells. WF-3 tumor cells served as target cells. WF-3 cells were mixed with splenocytes at various E:T ratios. Cytolysis was determined by quantitative measurements of LDH. Note: The pcDNA3-mesothelin DNA vaccine generated a significantly higher percentage of specific lysis than the other DNA vaccines (P<0.001). The data presented in this figure are from one representative experiment of two performed.

DETAILED DESCRIPTION OF THE INVENTION

[32]   The recent development of high-throughput technologies that quantify gene expression has led to the identification of many genes that are differentially expressed in human cancers. However, differential expression does not, on its own, indicate that an antigen is a therapeutic target. Therefore, a functional immunologic screen was applied to a SAGE gene expression database in order to identify immunologically relevant tumor antigens. We previously reported the association of prolonged disease-free survival and *in vivo* induction of anti-tumor immunity in three of fourteen patients receiving a pancreatic tumor vaccine. Here we identify mesothelin as a tumor antigen recognized by uncultured CD8+ T cells isolated from these vaccinated patients. Moreover, the induction of mesothelin-specific T cells was not found in the eleven other patients who received the same vaccine but relapsed. To validate mesothelin as a tumor antigen, we show that none of the patients respond to another differentially expressed gene product, prostate stem cell antigen. These data identify mesothelin as an *in vitro* marker of vaccine induced immune responses that correlate with clinical anticancer responses. The inventors also describe a functional genomic approach for identifying and validating other immunologically relevant human tumor antigens.

11

[33]   The vaccines of the present invention can be administered by any means known in the art for inducing a T cell cytolytic response. These means include oral administration, intravenous injection, percutaneous scarification, subcutaneous injection, intramuscular injection, and intranasal administration. The vaccines can be administered intradermally by gene gun. Gold particles coated with DNA may be used in the gene gun. Other inoculation routes as are known in the art can be used.

[34]   Additional agents which are beneficial to raising a cytolytic T cell response may be used as well. Such agents are termed herein carriers. These include, without limitation, B7 costimulatory molecule, interleukin-2, interferon-γ, GM-CSF, CTLA-4 antagonists, OX-40/OX-40 ligand, CD40/CD40 ligand, sargramostim, levamisol, vaccinia virus, Bacille Calmette-Guerin (BCG), liposomes, alum, Freund's complete or incomplete adjuvant, detoxified endotoxins, mineral oils, surface active substances such as lipolecithin, pluronic polyols, polyanions, peptides, and oil or hydrocarbon emulsions. Carriers for inducing a T cell immune response which preferentially stimulate a cytolytic T cell response versus an antibody response are preferred, although those that stimulate both types of response can be used as well. In cases where the agent is a polypeptide, the polypeptide itself or a polynucleotide encoding the polypeptide can be administered. The carrier can be a cell, such as an antigen presenting cell (APC) or a dendritic cell. Antigen presenting cells include such cell types aas macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used. Examples of facultative antigen-presenting cells include astrocytes, follicular cells, endothelium and fibroblasts. The carrier can be a bacterial cell that is transformed to express the polypeptide or to deliver a polynucleoteide which is subsequently expressed in cells of the vaccinated individual. Adjuvants, such as aluminum hydroxide or aluminum phosphate, can be added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences, like CpG, are also potential adjuvants. Other representative examples of adjuvants include the synthetic adjuvant QS-21 comprising a homogeneous saponin purified from the bark of *Quillaja saponaria* and *Corynebacterium parvum* (McCune et al., Cancer, 1979; 43:1619). It will be understood

that the adjuvant is subject to optimization. In other words, the skilled artisan can engage in routine experimentation to determine the best adjuvant to use.

[35]   Further additives, such as preservatives, stabilizers, adjuvants, antibiotics, and other substances can be used as well. Preservatives, such as thimerosal or 2-phenoxy ethanol, can be added to slow or stop the growth of bacteria or fungi resulting from inadvertent contamination, especially as might occur with vaccine vials intended for multiple uses or doses. Stabilizers, such as lactose or monosodium glutamate (MSG), can be added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process.

[36]   Viral vectors can be used to administer polynucleotides encoding a polypeptide comprising a mesothelin epitope. Such viral vectors include vaccinia virus and avian viruses, such as Newcastle disease virus. Others may be used as are known in the art.

[37]   One particular method for administering polypeptide vaccine is by pulsing the polypeptide onto an APC or dendritic cell *in vitro*. The polypeptide binds to MHC molecules on the surface of the APC or dendritic cell. Prior treatment of the APCs or dendritic cells with interferon-γ can be used to increase the number of MHC molecules on the APCs or dendritic cells. The pulsed cells can then be administered as a carrier for the polypeptide. Peptide pulsing is taught in Melero et al., *Gene Therapy* 7:1167 (2000).

[38]   Naked DNA can be injected directly into the host to produce an immune response. Such naked DNA vaccines may be injected intramuscularly into human muscle tissue, or through transdermal or intradermal delivery of the vaccine DNA, typically using biolistic-mediate gene transfer (*i.e.*, gene gun). Recent reviews describing the gene gun and muscle injection delivery strategies for DNA immunization include Tuting, Curr. Opin. Mol. Ther. (1999) 1: 216-25, Robinson, Int. J. Mol. Med. (1999) 4: 549-55, and Mumper and Ledbur, Mol. Biotechnol. (2001) 19: 79-95. Other possible methods for delivering plasmid DNA includes electroporation and iontophoreses.

[39]   Another possible gene delivery system comprises ionic complexes formed between DNA and polycationic liposomes (see, *e.g.*, Caplen et al. (1995) Nature Med. 1: 39). Held together by electrostatic interaction, these complexes may dissociate because of the charge screening effect of the polyelectrolytes in the biological fluid. A strongly basic

lipid composition can stabilize the complex, but such lipids may be cytotoxic. Other possible methods for delivering DNA includes electroporation and iontophoreses.

[40]   The use of intracellular and intercellular targeting strategies in DNA vaccines may further enhance the mesothelin-specific antitumor effect. Previously, intracellular targeting strategies and intercellular spreading strategies have been used to enhance MHC class I or MHC class II presentation of antigen, resulting in potent CD8+ or CD4+ T cell-mediated antitumor immunity, respectively. For example, MHC class I presentation of a model antigen, HPV-16 E7, was enhanced using linkage of Mycobacterium tuberculosis heat shock protein 70 (HSP70) (Chen, et al., (2000), Cancer Research, 60: 1035-1042), calreticulin (Cheng, et al., (2001) J Clin Invest, 108:669-678) or the translocation domain (domain II) of Pseudomonas aeruginosa exotoxin A (ETA(dII)) (Hung, et al., (2001) Cancer Research, 61: 3698-3703) to E7 in the context of a DNA vaccine. To enhance MHC class II antigen processing, the sorting signals of the lysosome associated membrane protein (LAMP-1) have been linked to the E7 antigen, creating the Sig/E7/LAMP-1 chimera (Ji, et al, (1999), Human Gene Therapy, 10: 2727-2740). To enhance further the potency of naked DNA vaccines, an intercellular strategy that facilitates the spread of antigen between cells can be used. This improves the potency of DNA vaccines as has been shown using herpes simplex virus (HSV-1) VP22, an HSV-1 tegument protein that has demonstrated the remarkable property of intercellular transport and is capable of distributing protein to many surrounding cells (Elliot, et al., (1997) Cell, 88: 223-233). Such enhanced intercellular spreading of linked protein, results in enhancement of antigen-specific CD8+ T cell-mediated immune responses and antitumor effect. Any such methods can be used to enhance DNA vaccine potency against mesothlin-expressing tumors.

[41]   Mesothelin is known to be expressed in ovarian cancer, pancreatic cancer, mesothelioma, and squamous cell carcinomas carcinomas of the esophagus, lung, and cervix. Thus the vaccines of the invention are useful for treating at least these types of tumors. Other tumors which express mesothelin can also be treated similarly.

[42]   In one embodiment, the vaccines of the present invention comprise a polypeptide comprising at least one MHC Class I-binding epitope of mesothelin or at least one MHC Class II-binding epitope of mesothelin. Alternatively, the vaccines of the present invention optionally comprise a polynucleotide encoding a polypeptide comprising at

least one MHC Class I-binding epitope of mesothelin or at least one MHC Class II-binding epitope of mesothelin. Optionally, the polypeptides of the vaccines (or the p olypeptides encoded by the polynucleotides of the vaccines) comprise a plurality of MHC Class I-binding epitopes of mesothelin and/or MHC Class II-binding epitopes of mesothelin. The multiple epitopes of the polypeptides may bind the same or different MHC allelic molecules. In one embodiment, the epitopes of the polypeptide bind a diverse variety of MHC allelic molecules.

[43]   While MHC Class I-binding epitopes are effective in the practice of the present invention, MHC Class II-binding epitopes can also be used. The former are useful for activating $CD8^+$ T cells and the latter for activating $CD4^+$ T cells. Publicly available algorithms can be used to select epitopes that bind to MHC class I and/or class II molecules. For example, the predictive algorithm "BIMAS" ranks potential HLA binding epitopes according to the predictive half-time disassociation of peptide/HLA complexes (23). The "SYFPEITHI" algorithm ranks peptides according to a score that accounts for the presence of primary and secondary HLA-binding anchor residues (25). Both computerized algorithms score candidate epitopes based on amino acid sequences within a given protein that have similar binding motifs to previously published HLA binding epitopes. Other algorithms can also be used to identify candidates for further biological testing.

[44]   Polypeptides for immunization to raise a cytolytic T cell response are optionally from 8 to 25 amino acid residues in length. Although nonamers are specifically disclosed herein, any 8 contiguous amino acids of the nonamers can be used as well. The polypeptides can be fused to other such epitopic polypeptides, or they can be fused to carriers, such as B-7, interleukin-2, or interferon-γ. The fusion polypeptide can be made by recombinant production or by chemical linkage, e.g., using heterobifunctional linking reagents. Mixtures of polypeptides can be used. These can be mixtures of epitopes for a single allelic type of an MHC molecule, or mixtures of epitopes for a variety of allelic types. The polypeptides can also contain a repeated series of an epitope sequence or different epitope sequences in a series.

[45]   The effectiveness of an MHC Class I-binding epitope of mesothelin or an MHC Class II-binding epitope of mesothelin as an immunogen in a vaccine can be evaluated by assessing whether a peptide comprising the epitope is capable of activating T-

lymphocytes from an individual having a successful immunological response to a tumor that overexpresses mesothelin (relative to normal tissue from which the tumor is derived), when the peptide is bound to an MHC molecule on an antigen-presenting cell and contacted with the T-lymphocytes under suitable conditions and for a time sufficient to permit activation of T-lymphocytes. A specific example of such an assessment is illustrated in Examples 1-4, below.

[46] Multiple groups have cloned cDNAs encoding mesothelin, and the sequences of the cDNA clones, as well as the sequence of the encoded mesothelin polypeptides, have been reported in U.S. Patent No. 6,153,430, Chang and Pastan, *Proc. Natl. Acad. Sci. USA*, 93:136-140 (1996), Kojima et al., *J. Biol. Chem.*, 270:21984-21990 (1995), and U.S. Patent No. 5,723,318. These references, including the sequences of the mesothelin-encoding nucleic acids, corresponding mesothelin polypeptides, and fragments described therein, are incorporated by reference herein in their entirety. Mesothelin cDNA encodes a protein with a molecular weight of approximately 69 kD, *i.e.*, the primary translation product. The 69 kD form of mesothelin is proteolytically processed to form a 40 kD mature mesothelin protein that is membrane-bound (Chang and Pastan (1996)). The term "mesothelin" as used herein encompasses all naturally occurring variants of the mesothelin, regardless of the cell or tissue in which the protein is expressed. In one embodiment, the mesothelin protein comprises one or more of the following amino acid sequences: SLLFLLFSL (SEQ ID NO:1); VLPLTVAEV (SEQ ID NO:2); ELAVALAQK (SEQ ID NO:3); ALQGGGPPY (SEQ ID NO:4); FYPGYLCSL (SEQ ID NO:5); and LYPKARLAF (SEQ ID NO:6). For instance, the mesothelin protein optionally comprises one, two, three, four, or five of these epitopes. In another embodiment, the mesothelin protein comprises each of the following amino acid sequences: SLLFLLFSL (SEQ ID NO:1); VLPLTVAEV (SEQ ID NO:2); ELAVALAQK (SEQ ID NO:3); ALQGGGPPY (SEQ ID NO:4); FYPGYLCSL (SEQ ID NO:5); and LYPKARLAF (SEQ ID NO:6).

[47] The vaccines of the invention optionally comprise mesothelin or a polynucleotide encoding mesothelin. For instance, the vaccine may comprise or encode the mature form of mesothelin, the primary translation product, or the full-length translation product of the mesothelin gene. In one embodiment, the vaccine comprises the cDNA of mesothelin. In addition to the use of naturally occurring forms of mesothelin (or polynucleotides encoding those forms), polypeptides comprising fragments of mesothelin, or

polynucleotides encoding fragments of mesothelin may be used in the vaccines. The polypeptides in the vaccines or encoded by polynucleotides of the vaccines are optionally at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, or at least about 50% identical to mesothelin.

[48]   In an alternative embodiment of the invention, the polypeptide of the vaccine or the polypeptide encoded by the polynucleotide of the vaccine is not a naturally-occurring mesothelin protein, such as the mature mesothelin protein, the primary translation product of mesothelin, or the mature megakaryocyte potentiating factor.

[49]   In one embodiment, the MHC Class I-binding epitope of mesothelin comprises less than 15 amino acids, less than 14 amino acids, less than 13 amino acids, less than 12 amino acids, or less than 11 amino acids in length. In another embodiment, the MHC Class I-binding epitope of mesothelin comprises at least seven or at least eight contiguous amino acids present in a peptide selected from the group consisting of SLLFLLFSL (SEQ ID NO:1), VLPLTVAEV (SEQ ID NO:2), ELAVALAQK (SEQ ID NO:3), ALQGGGPPY (SEQ ID NO:4), FYPGYLCSL (SEQ ID NO:5), and LYPKARLAF (SEQ ID. NO:6). The MHC Class I-binding epitope of mesothelin is at least 7 amino acids in length, at least 8 amino acids in length, or at least 9 amino acids in length.

[50]   In addition, the MHC Class I-binding epitopes of mesothelin and the MHC Class II binding epitopes of mesothelin used in vaccines of the present invention need not necessarily be identical in sequence to the naturally occurring epitope sequences within mesothelin. The naturally occurring epitope sequences are not necessarily optimal peptides for stimulating a CTL response. See, for example, (Parkhurst, M. R. et al., *J. Immunol.*, 157:2539-2548, (1996); Rosenberg, S. A. et al., *Nat. Med.*, 4:321-327, (1998)). Thus, there can be utility in modifying an epitope, such that it more readily induces a CTL response. Generally, epitopes may be modified at two types of positions. The epitopes may be modified at amino acid residues that are predicted to interact with the MHC molecule, in which case the goal is to create a peptide sequence that has a higher affinity for the MHC molecule than does the parent epitope. The epitopes can also be modified at amino acid residues that are predicted to interact with the T cell receptor on the CTL, in which case the goal is to create an epitope that has a higher affinity for the T cell receptor than does the parent epitope. Both of these types of modifications can result

in a variant epitope that is related to a parent eptiope, but which is better able to induce a CTL response than is the parent epitope.

[51]   Thus, the MHC Class I-binding epitopes of mesothelin identified in the Examples below (SEQ ID NO:1-6), or identified by application of the methods of the invention, and the MHC Class II-binding epitopes of mesothelin identified by application of the methods of the invention can be modified by the substitution of one or more residues at different, possibly selective, sites within the epitope sequence. Such substitutions may be of a conservative nature, for example, where one amino acid is replaced by an amino acid of similar structure and characteristics, such as where a hydrophobic amino acid is replaced by another hydrophobic amino acid. Even more conservative would be replacement of amino acids of the same or similar size and chemical nature, such as where leucine is replaced by isoleucine. In studies of sequence variations in families of naturally occurring homologous proteins, certain amino acid substitutions are more often tolerated than others, and these are often show correlation with similarities in size, charge, polarity, and hydrophobicity between the original amino acid and its replacement, and such is the basis for defining "conservative substitutions."

[52]   Conservative substitutions are herein defined as exchanges within one of the following five groups: Group 1--small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); Group 2--polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); Group 3--polar, positively charged residues (His, Arg, Lys); Group 4--large, aliphatic, nonpolar residues (Met, Leu, lie, Val, Cys); and Group 4--large, aromatic residues (Phe, Tyr, Trp). An acidic amino acid might also be substituted by a different acidic amino acid or a basic (i.e., alkaline) amino acid by a different basic amino acid. Less conservative substitutions might involve the replacement of one amino acid by another that has similar characteristics but is somewhat different in size, such as replacement of an alanine by an isoleucine residue.

[53]   Plasmids and viral vectors, for example, can be used to express a tumor antigen protein in a host cell. The host cell may be any prokaryotic or eukaryotic cell. Thus, for example, a nucleotide sequence derived from the cloning of mesothelin proteins, encoding all or a selected portion of the full-length protein, can be used to produce a recombinant form of a mesothelin polypeptide via microbial or eukaryotic cellular processes. The coding sequence can be ligated into a vector and the loaded vector can be used to transform or

transfect hosts, either eukaryotic (*e.g.*, yeast, avian, insect or mammalian) or prokaryotic (bacterial) cells. Such techniques involve standard procedures which are well known in the art.

[54]   Typically, expression vectors used for expressing a polypeptide, *in vivo* or *in vitro* contain a nucleic acid encoding an antigen polypeptide, operably linked to at least one transcriptional regulatory sequence. Regulatory sequences are art-recognized and can be selected to direct expression of the subject proteins in the desired fashion (time and place). Transcriptional regulatory sequences are described, for example, in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

[55]   Suitable vectors for the expression of a polypeptide comprising HLA-binding epitopes include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*. Mammalian expression vectors may contain both prokaryotic and eukaryotic sequences in order to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that can be expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Vaccinia and avian virus vectors can also be used. The methods which may be employed in the preparation of vectors and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

[56]   Other types of expression cassettes can also be used. For instance, the references described below in regard to viral, bacterial, and yeast vectors illustrate additional expression vectors which may be used in the present invention.

[57]   In another embodiment of the invention, a polypeptide described herein, or a polynucleotide encoding the polypeptide, is delivered to a host organism in an immunogenic composition comprising yeast. The use of live yeast DNA vaccine vectors for antigen delivery has been reviewed recently and reported to be efficacious in a mouse model using whole recombinant *Saccharomyces cerevisiae* yeast expressing tumor or HIV-1 antigens (see Stubbs et al. (2001) Nature Medicine 7: 625-29).

[58]   The use of live yeast vaccine vectors is known in the art. Furthermore, U.S. Patent No. 5,830,463, the contents of which are incorporated herein by reference, describes particularly useful vectors and systems for use in the instant invention. The use of yeast delivery systems may be particularly effective for use in the tumor/cancer vaccine methods and formulations of the invention as yeast appears to trigger cell-mediated immunity without the need for an additional adjuvant. Particularly preferred yeast vaccine delivery systems are nonpathogenic yeast carrying at least one recombinant expression system capable of modulating an immune response.

[59]   Bacteria can also be used as carriers for the epitopes of the present invention. Typically the bacteria used are mutant or recombinant. The bacterium is optionally attenuated. For instance, a number of bacterial species have been developed for use as vaccines and can be used in the present invention, including, but not limited to, *Shigella flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella typhimurium, Salmonella typhi* or mycobacterium. The bacterial vector used in the immunogenic composition may be a facultative, intracellular bacterial vector. The bacterium may be used to deliver a polypeptide described herein to antigen-presenting cells in the host organism. The use of live bacterial vaccine vectors for antigen delivery has been reviewed recently (Medina and Guzman (2001) Vaccine 19: 1573-1580; Weiss and Krusch, (2001) Biol. Chem. 382: 533-41; and Darji et al. (2000) FEMS Immunol and Medical Microbiology 27: 341-9). Furthermore, U.S. Patent Nos. 6,261,568 and 6,488,926, the contents of which are incorporated herein by reference, describe systems useful for cancer vaccines.

[60]   Bacterially mediated gene transfer is particularly useful in genetic vaccination by intramuscular, intradermal, or oral administration of plasmids; such vaccination leads to antigen expression in the vaccinee. Furthermore, bacteria can provide adjuvant effects and the ability to target inductive sites of the immune system. Furthermore, bacterial vaccine vectors have almost unlimited coding capacity. The use of bacterial carriers is

often associated with still other significant benefits, such as the possibility of direct mucosal or oral delivery. Other direct mucosal delivery systems (besides live viral or bacterial vaccine carriers) which can be used include mucosal adjuvants, viral particles, ISCOMs, liposomes, and microparticles.

[61]   Both attenuated and commensal microorganisms have been successfully used as carriers for vaccine antigens. Attenuated mucosal pathogens which may be used in the invention include: *L. monocytogenes, Salmonella* spp., *V. cholorae, Shigella* spp., mycobacterium, *Y. enterocolitica*, and *B. anthracis*. Commensal strains which can be used in the invention include: *S. gordonii, Lactobacillus* spp., and *Staphylococcus* spp. The genetic background of the carrier strain used in the formulation, the type of mutation selected to achieve attenuation, and the intrinsic properties of the immunogen can be adjusted to optimize the extent and quality of the immune response elicited. The general factors to be considered to optimize the immune response stimulated by the bacterial carrier include: selection of the carrier; the specific background strain, the attenuating mutation and the level of attenuation; the stabilization of the attenuated phenotype and the establishment of the optimal dosage. Other antigen-related factors to consider include: intrinsic properties of the antigen; the expression system, antigen-display form and stabilization of the recombinant phenotype; co-expression of modulating molecules and vaccination schedules.

[62]   *Salmonella typhimurium* can be used as a bacterial vector in the immunogenic compositions of the invention. Use of this bacterium as an effective vector for a vaccine has been demonstrated in the art. For instance, the use of *S. typhimurium* as an attenuated vector for oral somatic transgene vaccination has been described (see Darji et al. (1997) Cell 91: 765-775; and Darji et al. (2000) FEMS Immun and Medical Microbiology 27: 341-9). Indeed most knowledge of bacteria-mediated gene transfer has been acquired using attenuated *S. typhimurium* as carrier. Two metabolically attenuated strains that have been used include *S. typhimurium aroA*, which is unable to synthesize aromatic amino acids, and *S. typhimurium 22-11*, which is defective in purine metabolism. Several antigens have been expressed using these carriers: originally, listeriolysin and actA (two virulence factors of *L. monocytogenes*) and beta-galactosidase (*β-gal*) of *E. coli* were successfully tested. Cytotoxic and helper T cells as well as specific antibodies could be detected against these antigens following oral application of a single dose of the recombinant salmonella. In addition, immunization with *Salmonella* carrying a

listeriolysin-encoding expression plasmid elicited a protective response against a lethal challenge with *L. monocytogenes*. Oral transgene vaccination methodology has now been extended to include protective responses in herpes simplex virus 2 and hepatitis B infection models, with cell-mediated immune responses detected at the mucosal level.

[63]   In tumor models using *β-gal* as a surrogate tumor antigen, partial protective immunity against an aggressive fibrosarcoma was induced by orally administering *Salmonella* carrying a *β-gal*-encoding plasmid (see Paglia et al. (1998) Blood 92: 3172-76). In similar experiments using a *β-gal*-expressing transfectant of the murine renal cell carcinoma line RENCA, Zöller and Christ (Woo et al. (2001) Vaccine 19: 2945-2954) demonstrated superior efficacy when the antigen-encoding plasmid was delivered in bacterial carriers as opposed to using naked DNA. Interestingly, *Salmonella* can be used to induce a tumor growth retarding response against the murine melanoma B16; the *Salmonella* carry minigenes encoding epitopes of the autologous tumor antigens gp100 and TRP2 fused to ubiquitin. This suggests that under such circumstances peripheral tolerance towards autologous antigens can be overcome. This was confirmed by the same group (Lode et al. (2000) Med Ped Oncol 35: 641-646 using similar constructs of epitopes of tyrosine hydroxylase as autologous antigen in a murine neuroblastoma system. Furthermore, these findings were recently extended by immunizing mice that were transgenic for human carcinogenic antigen (hCEA) using a plasmid encoding a membrane-bound form of complete hCEA. In this case, a hCEA-expressing colon carcinoma system was tested and protection against a lethal challenge with the tumor could be improved by systemic application of interleukin 2 (IL-2) as adjuvant during the effector phase (see Xiang et al. (2001) Clin Cancer Res 7: 856s-864s).

[64]   Another bacterial vector which may be used in the immunogenic compositions described herein is *Salmonella typhi*. The *S. typhi* strain commonly used for immunization—*Ty21a galE*—lacks an essential component for cell-wall synthesis. Recently developed improved strains include those attenuated by a mutation in *guaBA*, which encodes an essential enzyme of the guanine biosynthesis pathway (Pasetti et al., Infect. Immun. (2002) 70:4009-18; Wang et al., Infect. Immun. (2001) 69:4734-41; Pasetti et al., Clin. Immunol. (1999) 92:76-89). Additional references describing the use of *Salmonella typhi* and/or other *Salmonella* strains as delivery vectors for DNA vaccines include the following: Lundin, Infect. Immun. (2002) 70:5622-7; Devico et al., Vaccine, (2002)

20:1968-74; Weiss et al., Biol. Chem. (2001) 382:533-41; and Bumann et al., FEMS Immunol. Med. Microbiol. (2000) 27:357-64.

[65]   The vaccines and immunogenic compositions of the present invention can employ *Shigella flexneri* as a delivery vehicle. *S. flexneri* represents the prototype of a bacterial DNA transfer vehicle as it escapes from the vacuole into the cytosol of the host cell. Several attenuated mutants of *S. flexneri* have been used successfully to transfer DNA to cell lines in vitro. Auxotrophic strains were defective in cell-wall synthesis (Sizemore et al. (1995) Science 270: 299-302 and Courvalin et al. (1995) C R Acad Sci Ser III, 318: 1207-12), synthesis of aromatic amino acids (Powell et al. (1996) Vaccines 96: Molecular Approaches to the Control of Infectious Disease; Cold Spring Harbor Laboratory Press) or synthesis of guanine nucleotides (Anderson et al. (2000) Vaccine 18: 2193-2202).

[66]   The vaccines and immunogenic compositions of the present invention can comprise *Listeria monocytogenes* (Portnoy et al, Journal of Cell Biology, 158:409-414 (2002); Glomski et al., Journal of Cell Biology, 156:1029-1038 (2002)). The ability of *L. monocytogenes* to serve as a vaccine vector has been reviewed in Wesikirch, et al., Immunol. Rev. 158:159-169 (1997). Strains of *Listeria monocytogenes* have recently been developed as effective intracellular delivery vehicles of heterologous proteins providing delivery of antigens to the immune system to induce an immune response to clinical conditions that do not permit injection of the disease-causing agent, such as cancer (U.S. Patent No. 6,051,237; Gunn et al., J. Of Immunology, 167:6471-6479 (2001); Liau, et al., Cancer Research, 62: 2287-2293 (2002); U.S. Patent No. 6,099,848; WO 99/25376; and WO 96/14087) and HIV (U.S. Patent No. 5,830,702). A recombinant *L. monocytogenes* vaccine expressing an lymphocytic choriomeningitis virus (LCMV) antigen has also been shown to induce protective cell-mediated immunity to the antigen (Shen et al., Proc. Natl. Acad. Sci. USA, 92: 3987-3991 (1995).

[67]   As a facultative intracellular bacterium, *L. monocytogenes* elicits both humoral and cell-mediated immune responses. Following entry of *Listeria* into a cell of the host organism, the *Listeria* produces *Listeria*-specific proteins that enable it to escape from the phagolysosome of the engulfing host cell into the cytosol of that cell. Here, *L. monocytogenes* proliferates, expressing proteins necessary for survival, but also expressing heterologous genes operably linked to *Listeria* promoters. Presentation of peptides of these heterologous proteins on the surface of the engulfing cell by MHC

proteins permit the development of a T cell response. Two integration vectors that are useful for introducing heterologous genes into the bacteria for use as vaccines include pL1 and pL2 as described in Lauer et al., Journal of Bacteriology, 184: 4177-4186 (2002).

[68]   In addition, attenuated forms of *L. monocytogenes* useful in immunogenic compositions have been produced. The ActA protein of *L. monocytogenes* is sufficient to promote the actin recruitment and polymerization events responsible for intracellular movement. A human safety study has reported that oral administration of an *actA/plcB*-deleted attenuated form of *Listeria monocytogenes* caused no serious sequelae in adults (Angelakopoulos et al., Infection and Immunity, 70:3592-3601 (2002)). Other types of attenuated forms of *L. monocytogenes* have also been described (see, for example, WO 99/25376 and U.S. Patent No. 6,099,848, which describe auxotrophic, attenuated strains of *Listeria* that express heterologous antigens).

[69]   *Yersinia enterocolitica* is another intraceullular bacteria that can optionally be used as a bacterial vector in immunogenic compositions of the present invention. The use of attenuated strains of *Yersini enterocolitica* as vaccine vectors is described in PCT Publication WO 02/077249.

[70]   In further embodiments of the invention, the immunogenic compositions of the invention comprise mycobacterium, such as Bacillus Calmette-Guerin (BCG). The Bacillus of Calmette and Guerin has been used as a vaccine vector in mouse models (Gicquel et al., Dev. Biol. Stand 82:171-8 (1994)). See also, Stover et al., Nature 351: 456-460 (1991).

[71]   Alternatively, viral vectors can be used. The viral vector will typically comprise a highly attenuated, non-replicative virus. Viral vectors include, but are not limited to, DNA viral vectors such as those based on adenoviruses, herpes simplex virus, avian viruses, such as Newcastle disease virus, poxviruses such as vaccinia virus, and parvoviruses, including adeno-associated virus; and RNA viral vectors, including, but not limited to, the retroviral vectors. Vaccinia vectors and methods useful in immunization protocols are described in U.S. Pat. No. 4,722,848. Retroviral vectors include murine leukemia virus, and lentiviruses such as human immunodeficiency virus. Naldini et al. (1996) Science 272:263-267. Replication-defective retroviral vectors harboring a polynucleotide of the invention as part of the retroviral genome can be used. Such vectors have been described

in detail. (Miller, et al. (1990) Mol. Cell Biol. 10:4239; Kolberg, R. (1992) J. NIH Res. 4:43; Cornetta, et al. (1991) Hum. Gene Therapy 2:215).

[72]　Adenovirus and adeno-associated virus vectors useful in this invention may be produced according to methods already taught in the art. (See, e.g., Karlsson, et al. (1986) EMBO 5:2377; Carter (1992) Current Opinion in Biotechnology 3:533-539; Muzcyzka (1992) Current Top. Microbiol. Immunol. 158:97-129; Gene Targeting: A Practical Approach (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible.

[73]　Alpha virus vectors, such as Venezuelan Equine Encephalitis (VEE) virus, Semliki Forest virus (SFV) and Sindbis virus vectors, can be used for efficient gene delivery. Replication-deficient vectors are available. Such vectors can be administered through any of a variety of means known in the art, such as, for example, intranasally or intratumorally. See Lundstrom, Curr. Gene Ther. 2001 1:19-29.

[74]　Additional references describing viral vectors which could be used in the methods of the present invention include the following: Horwitz, M. S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F. et al., pp. 109-128 in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, et al. (1995) FASEB Journal 9:190-199, Schreier (1994) Pharmaceutica Acta Helvetiae 68:145-159; Schneider and French (1993) Circulation 88:1937-1942; Curiel, et al. (1992) Human Gene Therapy 3:147-154; WO 95/00655; WO 95/16772; WO 95/23867; WO 94/26914; WO 95/02697 (Jan. 26, 1995); and WO 95/25071.

[75]　In another form of vaccine, DNA is complexed with liposomes or ligands that often target cell surface receptors. The complex is useful in that it helps protect DNA from degradation and helps target plasmid to specific tissues. The complexes are typically injected intravenously or intramuscularly.

[76]　Polynucleotides used as vaccines can be used in a complex with a colloidal dispersion system. A colloidal system includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of

DNA, e.g., with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (e.g., inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, et al., Ann NY Acad Sci 126-139, 1995). Formulation of DNA, e.g., with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, e.g., Canonico et al; Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268; Alton et al., Nat Genet. 5:135-142, 1993 and U.S. Patent No. 5,679,647.

[77] In addition, complex coacervation is a process of spontaneous phase separation that occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. The electrostatic interaction between the two species of macromolecules results in the separation of a coacervate (polymer-rich phase) from the supernatant (polymer-poor phase). This phenomenon can be used to form microspheres and encapsulate a variety of compounds. The encapsulation process can be performed entirely in aqueous solution and at low temperatures, and has a good chance, therefore, of preserving the bioactivity of the encapsulant. In developing an injectable controlled release system, the complex coacervation of gelatin and chondroitin sulfate to encapsulate a number of drugs and proteins has been exploited (see Truong, et al. (1995) Drug Delivery 2: 166) and cytokines have been encapsulated in these microspheres for cancer vaccination (see Golumbek et al. (1993) Cancer Res 53: 5841). Anti-inflammatory drugs have also been incorporated for intra-articular delivery to the joints for treating osteoarthritis (Brown et al. (1994) 331: 290). U.S. Patent Nos.: 6,193,970, 5,861,159 and 5,759,582, describe compositions and methods of use of complex coacervates for use as DNA vaccine delivery systems of the instant invention. In particular, U.S. Patent No. 6,475,995, the contents of which are incorporated herein by reference, teaches DNA vaccine delivery systems utilizing nanoparticle coacervates of nucleic acids and polycations which serve as effective vaccines when administered orally.

[78] Antibodies can be isolated which are specific for a particular MHC Class I- of Class II binding epitope of mesothelin. These antibodies may be monoclonal or polyclonal. They can be used, inter alia, for isolating and purifying polypeptides for use as vaccines. T-cell lines that bind to an MHC class I or class II-peptide complex comprising a particular MHC Class I- of Class II binding epitope of mesothelin are useful for screening for T cell adjuvants and immune response enhancers. Such cell lines can be isolated from patients

who have been immunized with a mesothelin-containing vaccine and who have mounted an effective T cell reseponse to mesothelin.

[79] To test candidate cancer vaccines in the mouse model, the candidate vaccine containing the desired tumor antigen can be administered to a population of mice either before or after challenge with the tumor cell line of the invention. Thus the mouse model can be used to test for both therapeutic and prophylactic effects. Vaccination with a candidate vaccine can be compared to control populations that are either not vaccinated, vaccinated with vehicle alone, or vaccinated with a vaccine that expresses an irrelevant antigen. If the vaccine is a recombinant microbe, its relative efficacy can be compared to a population of microbes in which the genome has not been modified to ecxpress the antigen. The effectiveness of candidate vaccine can be evaluated in terms of effect on tumor or ascites volume or in terms of survival rates. The tumor or ascites volume in mice vaccinated with candidate vaccine may be about 5%, about 10%, about 25%, about 50%, about 75%, about 90% or about 100% less than the tumor volume in mice that are either not vaccinated or are vaccinated with vehicle or a vaccine that expresses an irrevelant antigen. The differential in tumor or ascites volume may be observed at least about 10, at least about 17, or at least about 24 days following the implantation of the tumor cells into the mice. The median survival time in mice vaccinated with a nucleic acid-modified microbe may be, for example, at least about 2, at least about 5, at least about 7, or at least about 10 days longer than in mice that are either not vaccinated or are vaccinated with vehicle or a vaccine that expresses an irrelevant antigen.

[80] The mouse model can be used to test any kind of cancer treatment known in the art. These may be conventional or complementry medicines. These can be immunological agents or cytotoxic agents. For example, the candidate cancer treatment may be radiation therapy, chemotherapy, or surgery. The candidate cancer treatment may be a combination of two or more therapies or prophylaxes, including but not limited to anti-cancer agents, anti-tumor vaccines, radiation therapy, chemotherapies, and surgery.

[81] Any oncogene known in the art can be used to make the peritoneal or mesothelium cell line for making the mouse model. Such oncogenes include without limitation, *Ki-ras, Erb-B2, N-ras, N-myc, L-myc, C-myc, ABL1, EGFR, Fos, Jun, c-Ha-ras, and SRC.*

[82] The vaccines, polynucleotides, polypeptides, cells, and viruses of the present invention can be administered to either human or other mammals. The other mammals can be

domestic animals, such as goats, pigs, cows, horses, and sheep, or can be pets, such as dogs, rabbits, and cats. The other mammals can be experimental subjects, such as mice, rats, rabbits, monkeys, or donkeys.

[83]   A reagent used in therapeutic methods of the invention is present in a pharmaceutical composition. Pharmaceutical compositions typically comprise a pharmaceutically acceptable carrier, which meets industry standards for sterility, isotonicity, stability, and non-pyrogenicity and which is nontoxic to the recipient at the dosages and concentrations employed. The particular carrier used depends on the type and concentration of the therapeutic agent in the composition and the intended route of administration. If desired, a stabilizing compound can be included. Formulation of pharmaceutical compositions is well known and is described, for example, in U.S. Patents 5,580,561 and 5,891,725.

[84]   The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient that increases anti-tumor cytolytic T-cell activity relative to that which occurs in the absence of the therapeutically effective dose.

[85]   For any substance, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

[86]   Therapeutic efficacy and toxicity, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

[87]   Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

28

[88]   The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors that can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

[89]   Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc. Effective in vivo dosages of polynucleotides and polypeptides are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 μg to about 2 mg, about 5 μg to about 500 μg, and about 20 μg to about 100 μg.

[90]   Desirable immunogens for use as anti-tumor vaccines are those which are highly differentially expressed between tumors and their corresponding normal tissues. Expression differences are preferably at least 2-fold, 3-fold, 4-fold, 5-fold, or even 10 fold. Expression can be measured by any means known in the art, including but not limited to SAGE, microarrays, Northern blots, and Western blots. Interest in such proteins as immunogens is enhanced by determining that humans respond to immunization with the protein (or gene encoding it) by generating CD4 or CD8 T cells which are specifically activated by the protein. Testing for such activation can be done, inter alia, using TAP deficient cell lines such as the human T2 cell line to present potential antigens in an MHC complex. Activation can be measured by any assay known in the art. One such assay is the ELISPOT assay. See references 33-35.

[91]   Future responses to tumor vaccines can be predicted based on the response of CD8+ and or CD4+ T cells. If the tumor vaccine comprises mesothelin or at least one T cell epitope

of mesothelin, then monitoring of the of CD8+ and or CD4+ response to mesothelin provides useful prognostic information. A robust CD8+ and or CD4+ response indicates that the patient has mounted an effective immunological response and will survive significantly longer than those who have not mounted such a response. The tumor vaccine may comprise whole tumor cells, particularly pancreatic, ovarian or mesothelioma cells. The tumor vaccine may comprise a polyethylene glycol fusion of tumor cells and dendritic cells. The tumor vaccine may comprise apoptotic or necrotic tumor cells which have been incubated with dendritic cells. The tumor vaccine may comprise mRNA or whole RNA which has been incubated wioth dendritic cells. The T cell responses to mesothelin can be measured by any assay known in the art, including an ELISPOT assay. Alternatively, future response to such a tumor vaccine can be monitored by assaying for a delayed type hypersensitivity respone to mesothelin. Such a response has been identified as a positive prognostic indicator.

[92]   Test substances which can be tested for use as a potential drug or immune enhancing agent can be any substance known in the art. The substance can be previously known for another purpose, or it can be previously unknown for any purpose. The substance can be a purified compound, such as a single protein, nucleic acid, or small molecule, or it can be a mixture, such as an extract from a natural source. The substance can be a natural product, or it can be a synthetic product. The substance can be specifically and purposefully synthesized for this purpose or it can be a substance in a library of compounds which can be screened.

[93]   While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the spirit and scope of the invention as set forth in the appended claims.

EXAMPLES

Example 1

[94]   To identify genes that can serve as potential immune targets for the majority of pancreatic adenocarcinoma patients, we focused only on those genes that were non-mutated, overexpressed by the majority of pancreatic cancer patients, and overexpressed by the vaccine cell lines. One gene at the top of this list was mesothelin (20, 21). For comparison and validation purposes we also looked at prostate stem cell antigen (PSCA). SAGE data demonstrated PSCA to be expressed by pancreatic tumors at similar levels to that of mesothelin (22).

[95]   We used the combination of two public use computer algorithms (23-25) to predict peptide nonamers that bind to three common human leukocyte antigen (HLA)-class I molecules. All 14 patients treated with the allogeneic GM-CSF vaccine express at least one of these HLA-Class I molecules (Table 2). The predictive algorithm "BIMAS", ranks potential HLA binding epitopes according to the predictive half-time disassociation of peptide/HLA complexes (23). The "SYFPEITHI" algorithm ranks peptides according to a score that accounts for the presence of primary and secondary HLA-binding anchor residues (25). Both computerized algorithms score candidate epitopes based on amino acid sequences within a given protein that have similar binding motifs to previously published HLA binding epitopes. We synthesized the top two ranking mesothelin epitopes for HLA-A2, HLA-A3, and HLA-A24 and the top six PSCA epitopes for each MHC molecule favored by both algorithms (Table 1), since at least one of these three HLA class I molecules is expressed by the 14 patients that were treated in our vaccine study (Table 2).

Table 1. Mesothelin peptides predicted to bind to HLA A2, A3, and A24.

HLA-Restriction	Amino Acid Sequence	Amino Acid Position in Protein
HLA-A2	SLLFLLFSL	Mesothelin A2$_{(20-28)}$
HLA-A2	VLPLTVAEV	Mesothelin A2$_{(530-538)}$
HLA-A2	LLALLMAGL	PSCA A2$_{(5-13)}$
HLA-A2	ALQPGTALL	PSCA A2$_{(14-22)}$
HLA-A2	ALLPALGLL	PSCA A2$_{(108-116)}$
HLA-A3	ELAVALAQK	Mesothelin A3$_{(83-92)}$
HLA-A3	ALQGGGPPY	Mesothelin A3$_{(225-234)}$
HLA-A3	ALQPAAAIL	PSCA A3$_{(99-107)}$
HLA-A3	LLALLMAGL	PSCA A3$_{(5-13)}$
HLA-A3	ALQPGTALL	PSCA A3$_{(14-22)}$
HLA-A3	LLPALGLLL	PSCA A3$_{(109-117)}$
HLA-A3	QLGEQCWTA	PSCA A3$_{(43-51)}$
HLA-A3	ALLCYSCKA	PSCA A3$_{(20-28)}$
HLA-A24	FYPGYLCSL	Mesothelin A24$_{(435-444)}$
HLA-A24	LYPKARLAF	Mesothelin A24$_{(475-484)}$
HLA-A24	DYYVGKKNI	PSCA A24$_{(76-84)}$
HLA-A24	ALLPALGLL	PSCA A24$_{(108-116)}$
HLA-A24	ALQPAAAIL	PSCA A24$_{(99-107)}$
HLA-A24	LLPALGLLL	PSCA A24$_{(109-117)}$
HLA-A24	YYVGKKNIT	PSCA A24$_{(77-85)}$

The three peptides, HIV-gag A2$_{77-85}$ (SLYNTVATL) (48), HIV-NEF A3$_{94-103}$ (QVPLRPMTYK) (49), and tyrosinase A24$_{206-214}$ (AFLPWHRLF) (50), are previously published epitopes that were used as control peptides for HLA-A2, A3, and A24 binding, respectively. The Mesothelin A1$_{309-318}$ binding epitope (EIDESLIFY) was used as a negative control peptide for all binding studies. The M1 peptide (GILGFVFTL)$_{58-66}$ (Gotch et al 1988) was used as a positive control for all of the HLA-A2 studies.

Table 2. Selected Characteristics of the 14 patients treated with an allogeneic GM-CSF secreting pancreatic tumor vaccine.

Pt #	Disease Status			Dose X10[7] Cells	# of total vaccines received	HLA Class I Expression at the A Locus[1]	Post-vaccine increase in DTH to Auto Tumor[2]	Disease-free Survival (months)	Overall Survival (months)
	T (cm)	LN	Margins						
1	3.0	5/17	-	1	2	A1, A2	0	11	14
2	2.7	3/17	+	1	1	A2, A3	0	6	14
3	2.5	3/11	+	1	1	A1, A3	0	9	18
4	2.5	3/14	-	5	1	A2, A29	0	8	10
5	2.7	2/23	-	5	4	A3, A3	0	15	39
6	2.5	2/17	-	5	3	A2, A24	0	13	27
7	4.0	4/13	+	10	1	A31, A24	0	16	18
8	2.7	5/18	+	10	2	A2, A3	252 mm	60+	60+
9	1.2	2/11	-	10	1	A3, A31	0	8	21
10	2.0	11/27	-	50	1	A3, A30	0	9	17
11	3.5	2/32	+	50	1	A1, A3	N/A	9	13
12	2.5	2/11	-	50	1	A3, A33	N/A	11	13
13	3.0	2/14	-	50	4	A3, A23	100 mm	60+	60+
14	3.0	0/14	+	50	4	A1, A24	110 mm	60+	60+

Abbreviations: Pt=patient, #=number, T=tumor size at surgery, LN=number of positive lymph nodes/total number of lymph nodes sampled, HLA=human leukocyte antigen, DTH=delayed type hypersensitivity testing, Auto=autologous, N/A=not assessed due to unavailability of DTH cell reagents, +=still alive and disease-free.

[1]HLA typing was performed serologically and confirmed molecularly.

[2]Delayed type hypersensitivity reactions to autologous tumor cells was assessed using unpassaged autologous tumor cells. 10^6 autologous tumor cells were placed pre-vaccination, and at 28 days post-vaccination. Reported are the post-vaccination change in the product of the perpendicular diameters (measured in mm) of the observed induration at 48 hours after cell placement.

[96]   Binding of these epitopes to their respective HLA class I molecule was tested by pulsing
        TAP deficient T2 cells that expressed the corresponding HLA class I molecule (T2-A2,
        T2-A3, or T2-A24 cells).   As shown in Fig. 1A, pulsing of two mesothelin-derived
        epitopes predicted to bind to HLA-A2 allows for detection of HLA-A2 on the cell surface
        of T2-A2 cells by flow cytometry following staining with the HLA class I specific
        antibody, W6/32. In contrast, unpulsed T2 cells or T2 cells pulsed with an mesothelin
        epitope predicted to bind to HLA-A1 do not stain with the same antibody. Binding of T2
        cells pulsed with two candidate mesothelin derived HLA-A3 and two candidate HLA-
        A24 epitopes are shown in Fig. 1B and Fig. 1C, respectively. A similar binding
        experiment was done with the PSCA derived peptides for HLA-A2, HLA-A3, and HLA-
        A24. (Fig. 1D, Fig. 1E and Fig. 1F).

[97]   Materials and Methods: Identification of candidate genes and epitope selection. SAGE
        was used to identify mesothelin as one of the genes overexpressed in pancreatic cancer
        cell lines and fresh tissue as previously reported (20, 21). Two computer algorithms that
        are available to the general public and accessible through the internet   were used to
        predict peptides that bind to HLA A2, A3, and A24 molecules. "BIMAS" was developed
        by K.C. Parker and collaborators http://bimas.dcrt.nih.gov/ (NIH) that determined the
        optimal binding for the most common HLA class I molecule types (23). "SYFPEITHI"
        was developed by Rammensee et al. and ranks the peptides according to a score that takes
        into account the presence of primary and secondary MHC-binding anchor residues
        http://www.uni-tuebingen.de/uni/kxi (24).

[98]   Materials and Methods: Peptides and T2 cell lines. Peptides were synthesized by
        Macromolecular Resources (Fort Collins, Colorado) according to published sequences:
        M1 peptide GILGFVFTL (SEQ ID NO: 10), derived from influenza matrix protein
        (amino acid positions 58-66) (28), Mesothelin A2 peptides and PSCA A2 peptides listed
        in table 1 were identified using the available databases, HIV-gag A2 peptide
        SLYNTVATL (SEQ ID NO: 7) (amino acid positions 75-83) (29) contain an HLA-A2
        binding motif. Mesothelin A3 peptides and PSCA A3 peptides and HIV-NEF A3 peptide
        QVPLRPMTYK (SEQ ID NO: 8) (amino acid positions 94-103) (30) contain an HLA-
        A3 binding motif. Mesothelin A24 peptides and PSCA A24 peptides and Tyrosinase
        peptide AFLPWHRLF (SEQ ID NO: 9) (amino acid positions 206-214) (31) contain an

HLA-A24 binding motif. Stock solutions (1mg/ml) of each peptide were prepared in 10%DMSO (JTBaker, Phillippsburg, NJ) and further diluted in cell culture medium to yield a final peptide concentration of 10ng/ml for each assay. The control M1 peptide was initially dissolved in 100% DMSO and further diluted in cell culture medium using the same stock and final concentrations. The T2 cells are a human B and T lymphoblast hybrid that only express the HLA-A*0201 allele (26). The human T2 cell line is a TAP deficient cell line that fails to transport newly digested HLA class I binding epitopes from the cytosol into the endoplasmic reticulum where these epitopes would normally bind to nascent HLA molecules and stabilize them for expression on the cell surface (26). The T2 -A3 are T2 cells genetically modified to express the HLA-A301 allele and were a gift from Dr. Walter Storkus (University of Pittsburgh) (32). T2-A24 are T2 cells genetically modified to express the HLA-A24 allele. The HLA-A24 gene was a gift from Dr. Paul Robbins (Surgery Branch, National Cancer Institute) (31). T2 cells were grown in suspension culture in RPMI-1640 (Gibco, Grand Island, NY), 10% serum (Hyclone, Logan, Utah) supplemented with 200μM L-Glutamine (Gibco, Grand Island, NY), 50 units-μg/ml Pen/Strep (Gibco), 1% NEAA (Gibco), and 1% Na-Pyruvate (Gibco).

[99] Materials and Methods: Peptide/MHC binding Assays. T2 cells expressing the HLA molecule of interest were resuspended in AimV serum free media (Gibco) to a concentration of 2.5×10^5cells/ml and pulsed with 100-200 micrograms of peptide at room temperature overnight. Pulsing at room temperature allows for optimizing the number of empty HLA molecules available for binding each epitope (30). The cells were washed and resuspended at 1×10^5cells/ml. Peptide binding was determined by FACS (Beckon Dickenson, San Jose, CA) analysis.

Example 2

[100] To determine if mesothelin and PSCA are recognized by CD8+ T cells, we screened antigen-pulsed T2 cells with CD8+ T cell enriched PBL from patients that have received an allogeneic GM-CSF secreting pancreatic tumor vaccine. We previously reported the association of in vivo post-vaccination delayed type hypersensitivity (DTH) responses to autologous tumor in three of eight patients receiving the highest two doses of vaccine. These "DTH responders" (each of whom had poor prognostic indicators at the time of primary surgical resection (27) are the only patients who remain clinically free of pancreatic cancer > 4 years after diagnosis ((27), Table 2). PBL obtained prior to

vaccination and 28 days after the first vaccination were initially analyzed. T2-A3 cells pulsed with the two A3 binding epitopes were incubated overnight with CD8+ T cell enriched lymphocytes isolated from the peripheral blood of patient 10 (non-DTH responder who relapsed 9 months after diagnosis) and 13 (DTH responder who remains disease-free) and analyzed using a gamma interferon (IFN-γ) ELISPOT assay. The ELISPOT assay was chosen because it requires relatively few lymphocytes, is among the most sensitive in vitro assays for quantitating antigen-specific T cells, and correlates number of antigen-specific T cells with function (cytokine expression) (33-35). The number of IFN-γ spots per 1x105 CD8+ positive T cells detected in the peripheral blood of the two patients prior to vaccination and twenty-eight days following the first vaccination in response to the two HLA-A3 binding mesothelin peptides are shown in Fig. 2A.

[101] Induction of mesothelin-specific T cells was detected twenty-eight days following vaccination in patient 13 a DTH responder, but not in patient 10, a non-DTH responder. Similarly, post-vaccination induction of mesothelin-specific CD8+ T cells was observed in two other disease-free DTH responders (patient 8 and patient 14), but not for two other non-DTH responders when tested with T2-A2 and T2-A24 cells pulsed with the A2 (Fig. 2B) and A24 (Fig. 2C) binding epitopes, respectively. A summary of the ELISPOT results analyzing all 14 patients treated with the allogeneic vaccine on this study for the induction of mesothelin-specific CD8+ T cells following the first vaccination are shown in Fig. 2D. These data demonstrate that there is a direct correlation between observed post-vaccination in vivo DTH responses to autologous tumor, long term disease-free survival, and post-vaccination induction of mesothelin-specific T cell responses in this clinical trial. Specifically, each of the three DTH responders demonstrated a post-vaccination induction in T cell response to every mesothelin peptide that matched their respective HLA type, whereas only one of eleven DTH non-responders had an increased post-vaccination mesothelin-specific T cell response and only to a single peptide. Thus, the in vitro measurement of mesothelin-specific T cells responses represents a new candidate in vitro immune marker for predicting which patients will respond to this vaccine therapy.

[102] Materials and Methods: Peripheral blood lymphocytes (PBL) and donors. Peripheral blood (100cc pre-vaccination and 28 days after each vaccination) were obtained from all fourteen patients who received an allogeneic GM-CSF secreting pancreatic tumor vaccine

as part of a previously reported phase I vaccine study (27).   Informed consent for banking lymphocytes to be used for this antigen identification study was obtained at the time of patient enrollment into the study. Pre and post-vaccine PBL were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were washed twice with serum free RPMI-1640. PBL were stored frozen at -180°C in 90% AIM-V media containing 10% DMSO.

[103] Materials and Methods: Enrichment of PBL for CD8+ T cells. CD8+ T cells were isolated from thawed PBL using Magnetic Cell Sorting of Human Leukocytes as per the manufacturers directions(MACS, Miltenyi Biotec, Auburn, CA).   Cells were fluorescently stained with CD8-PE antibody (Becton Dickenson, San Jose, CA) to confirm that the positive population contained CD8+ T cells and analyzed by flow cytometry. This procedure consistently yielded >95% CD8+ T cell purity.

[104] Materials and Methods: ELISPOT assay. Multiscreen ninety-six well filtration plates (Millipore, Bedford, MA) were coated overnight at 4°C with 60μl/well of 10μg/ml anti-hIFN-γ mouse monoclonal antibody (Mab) 1-D1K (Mabtech, Nacka, Sweden). Wells were then washed 3 times each with 1xPBS and blocked for 2 hours with T cell media. 1x105 T2 cells pulsed with peptide (10ng/ml) in 100μl of T cell media were incubated overnight with 1x105 thawed PBL that are purified to select CD8+ T cells in 100μl T-cell media on the ELISPOT plates in replicates of six. The plates were incubated overnight at 37°C in 5% CO2. Cells were removed from the ELISPOT plates by washing six times with PBS + 0.05% Tween 20 (Sigma, St. Louis, MO). Wells were incubated for 2 hours at 37°C in 5% CO2 using 60μl/well of 2μg/ml biotinylated Mab anti-hIFNgamma 7-B6-1 (Mabtech, Nacka, Sweden). The avidin peroxidase complex (Vectastain ELITE ABC kit, Vetcor Laboratories, Burlingame, CA) was added after washing six times with PBS/Tween 0.05% at 100μl per well and incubated for one hour at room temperature. AEC-substrate solution   (3-amino-9-ethylcarbazole) was added at 100μl/well and incubated for 4-12 minutes at room temperature. Color development was stopped by washing with tap water. Plates were dried overnight at room temperature and colored spots were counted using an automated image system ELISPOT reader (Axioplan2, Carl Zeiss Microimaging Inc., Thornwood, NY).

. Example 3

[105]   The above data clearly demonstrate a correlation of *in vivo* DTH response to autologous tumor and long term disease-free survival with the post-vaccination induction of mesothelin-specific CD8+ T cell responses. It is possible, however, that this correlation represents generalized immune suppression (in the patients who failed to demonstrate post-vaccination DTH responses to their autologous tumor and who had disease progression), rather than a vaccine specific induction of T cell responses to mesothelin in the DTH responder patients who remain disease-free. To demonstrate that the post-vaccination induction of mesothelin-specific CD8+ T cells is tumor antigen-specific, we evaluated each HLA-A2 positive patient for T cell responses to the HLA-A2-binding influenza matrix peptide, M1 (28). We chose the influenza M1 peptide because most patients on the vaccine study had received an influenza vaccine sometime prior to enrollment. As shown in Fig. 3, all HLA-A2 positive patients demonstrated similar pre- and post-vaccination T cell responses to the M1 peptide. Pre-vaccination responses ranged from 19 to 50 IFN-γ spots per 105 total CD8+ T cells, and post-vaccination responses remained about the same in each patient (Fig. 3). A similar study was not done for HLA-A3 and A24 positive patients because there are no published influenza M1 epitopes known to bind these HLA molecules.

[106]   We evaluated the lymphocytes from the same 14 patients for the post-vaccination induction of CD8+ T lymphocytes directed against a second overexpressed antigen, PSCA. In contrast to mesothelin, PSCA did not elicit an immune response in the 3 DTH responders. Again, we synthesized the top two ranking epitopes for HLA-A2, HLA-A3, and HLA-A24 favored by both algorithms and analyzed these according to the same protocols used in the mesothelin experiments. We did not see any post-vaccination induction of PSCA-specific T cells in any of the patients; therefore, we synthesized 4 additional PSCA peptides for each HLA class I molecule to ensure that we had not missed the immunogenic epitope. Analysis of these peptides also failed to demonstrate a post-vaccination induction of PSCA-specific CD8+ T cell responses (Figs. 4A, 4B, and 4C, respectively). PSCA specific responses could not be demonstrated in the eight non-responders as well (Fig. 4d). This result further supports our finding that mesothelin is a relevant pancreatic tumor antigen because there were no vaccine induced immune responses to PSCA even though they are similarly overexpressed in pancreatic cancer on

38

SAGE analysis. In addition, the PSCA data demonstrate that overexpression of a protein in a tumor is insufficient to predict the protein's utility as a vaccine target.

[107] Flow cytometry analysis of mesothelin and PSCA expression by the two allogeneic vaccine cell lines is shown in Fig. 5. Interestingly, mesothelin is expressed equally by both vaccine cell lines whereas PSCA is only expressed by one of the vaccine cell lines (Panc 6.03).

[108] Materials and Methods: CD8+ M1 specific T cell lines. M1 specific T cell lines were generated by repeated in vitro stimulation of HLA-A201 positive PBL initially with irradiated autologous dendritic cells followed by irradiated autologous Ebstein Barr Virus (EBV) transformed B cells, both pulsed with the HLA-A201 restricted epitope. This line was stimulated biweekly using autologous EBV cells that were pulsed with 10 μg peptide/ml of their respective peptides at 37°C for 2 hours, washed twice with RPMI-1640, and irradiated with 10,000 rads. T cells were stimulated at a 1:2 T cell to EBV cell ratio in T cell media (RPMI-1640, 10% human serum (pooled serum collected at the Johns Hopkins Hemapheresis Unit) containing 200μM L-Glutamine, 50 units-μg/ml Pen/Strep, 1% NEAA, and 1% Na-Pyruvate) supplemented with 20 cetus units IL-2/well and 10ng/well IL-7. This line was used a positive control T cell line in all assays.

[109] Materials and Methods: Flow cytometry. The expression of mesothelin and PSCA on the vaccine lines was evaluated by flow cytometry analysis. The vaccine lines were washed twice and resuspended in "FACS" buffer (HBSS supplemented with 1% PBS, 2% FBS, and 0.2% sodium azide), then stained with mouse monoclonal mesothelin (CAK1) (Signet Laboratories, Dedham, MA) or mouse monoclonal to PSCA (clone 1G8, obtained from R.E.R.) followed by FITC-labeled goat antimouse IgG (BD PharMingen, San Jose, CA) for flow analysis in a FACScan analyzer (BD Immunocytometry Systems).

[110] These data demonstrate that mesothelin-specific CD8+ T cells are detected following a single vaccination with an allogeneic GM-CSF secreting tumor vaccine in DTH responders but not in non-DTH responders. The patients treated on the reported vaccine study received an initial vaccination 8-10 weeks following pancreaticoduodenectomy and 4 weeks prior to receiving a six month course of adjuvant chemoradiation (27). Six of these patients remained disease-free at the end of the six months and received up to 3

39

more vaccinations given one month apart. Repeat ELISPOT studies were performed on serial CD8+ T cell enriched PBL samples from these six patients following multiple vaccine treatments to assess the effect of chemoradiation and multiple vaccinations on mesothelin-specific T cell responses.

[111]   As shown in Fig. 6, two of the three DTH responders demonstrated decreased mesothelin-specific T cell responses following the second vaccination. In both patients, mesothelin-specific T cell responses returned to levels achieved after the initial vaccination by the fourth vaccination. The suppressed mesothelin-specific T cell responses that were observed following the second vaccine are likely the result of the chemotherapy that each patient received between the first and second vaccination. Interestingly, one of the three patients demonstrated similar mesothelin-specific T cell responses after the first and second vaccination. This DTH responder only received two vaccines because she subsequently developed a late autoimmune antibody mediated complication attributed to the Mitomycin-C that required medical intervention and withdrawal from the vaccine study. In contrast, repeated vaccination failed to induce mesothelin-specific T cell responses in those patients who did not demonstrate an initial mesothelin-specific T cell response following the first vaccination (Fig. 6).

[112]   These data describing CD8$^+$ T cell responses induced by an allogeneic GM-CSF-secreting pancreatic tumor vaccine support the following conclusions. First, mesothelin can serve as an *in vitro* biomarker of vaccine-induced immune responses that correlate with *in vivo* responses in patients with pancreatic adenocarcinoma. Second, the recognition of mesothelin and lack of recognition of another overexpressed gene product, PSCA, by uncultured post-vaccination CD8$^+$ T cells from patients that demonstrated evidence of *in vivo* immune responses in association with clinical responses validates this antigen identification approach as a rapid functional genomic-based approach for identifying immune relevant tumor targets of CD8$^+$ T cells.

[113]   Mesothelin is a 40 kilodalton transmembrane glycoprotein member of the mesothelin/megakaryocyte potentiating factor (MPF) family expressed by most pancreatic adenocarcinomas (36), (37-39). It has also been reported to be expressed by ovarian cancers, mesotheliomas, and some squamous cell carcinomas (37-39). Mesothelin is known to be attached to the cell membrane by a glycosylphosphatidyl-inositol anchor and is postulated to function in cell adhesion (36). Mesothelin and other

members of its gene family exhibit limited normal tissue expression. It therefore meets three important criteria that strongly favor its potential use as an immunogen in the future development of antigen-based vaccines for patients with pancreatic adenocarcinoma and other tumor types that overexpress mesothelin: it is widely shared by most pancreatic and ovarian cancers, it has a limited expression in normal tissues, and it induces CD8$^+$ T cell responses following vaccination with tumor cells that express this antigen.

[114] The identification of shared, biologically relevant tumor antigens provides the opportunity to design antigen-based vaccines that have the potential to be more efficient at inducing anti-tumor immunity than current whole cell vaccines. In addition, scale up of recombinant antigen-based approaches is technically more feasible than currently employed whole tumor cell vaccines. However, recombinant antigen-based vaccines require identification of antigens that are both broadly expressed by patients and that are immunogenic. Until now, T cell screening of cDNA libraries, antibody screening of phage display libraries, or the biochemical elution and purification of antigens bound to MHC have identified the majority of known tumor antigens, many of which appear to derive from shared, non-mutated genes that are either overexpressed by or reactivated in tumor cells relative to normal tissue (3-13). Unfortunately, this expanding list of tumor associated antigens recognized by T cells is limited mostly to melanoma because of the technical difficulty of isolating and propagating T cell lines and clones from vaccinated patients with other types of cancer. The tumor antigen identification approach disclosed herein is feasible because it only requires a database of differentially expressed genes within a given tumor, and banked, uncultured bulk PBL from vaccinated patients. Therefore, this antigen identification approach is rapid and can be generalized to most types of cancer. In addition, the use of uncultured lymphocytes rather than T cell lines and clones that have been in long term culture provide the advantage of identifying new biologically relevant immune targets.

[115] PSCA is a second gene product that was found to be overexpressed in our SAGE pancreatic gene expression database. In fact, PSCA was shown to be overexpressed at higher levels than even mesothelin. However, post-vaccination PSCA specific T cell responses were not detected in the DTH responders and DTH non-responder patients. It is unclear at this time why a GM-CSF secreting allogeneic vaccine induces T cell responses to one overexpressed antigen and not to a second similarly overexpressed

antigen. It is possible that these two antigens are differently processed and presented during the initial priming event (58).

[116] In this study we also demonstrate that mesothelin-specific T cells can be induced against at least six different peptides presented by three different HLA-A locus alleles. This finding provides further support that mesothelin can serve as a shared antigen. In this study, the highest ranking antigenic epitopes predicted to be the best HLA-A allele binding epitopes based on their motif, bound to their respective HLA alleles and were also recognized by mesothelin-specific T cells. Reports analyzing other tumor antigens have found that the highest ranking epitopes do not necessarily correlate with optimal recognition by T cells (25). We also performed the computer algorithms on two melanoma antigens, tyrosinase and MAGE 1, to determine how their published HLA-A2 binding peptides rank by this method. We found that our HLA-A2 binding mesothelin epitopes were given similar scores as the known tyrosinase and MAGE 1 HLA-A2 binding epitopes. This was also true for the published HLA-A2 HIV GAG and HLA-A3 HIV NEF epitopes that were used as control antigens in our analyses. Choosing epitopes that rank high by both algorithms appears to be an important predictor of the probability of binding to the respective HLA molecule.

[117] We have developed a functional genomic approach that identified a candidate pancreatic tumor antigen. This approach to antigen identification facilitates the identification of other human cancer antigens that are biologically relevant immune targets. The correlation of *in vitro* T cell responses with *in vivo* measures of response validates the biologic importance of this approach. This approach is rapid and feasible and can easily be adapted to identify antigens expressed by other cancer types. This in turn, should accelerate the development of recombinant antigen-based vaccines for most human cancer treatment.

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Example 5

[118]   Construction of Mouse Tumor Cells by Co-transformation with HPV-16 E6 and E7 and
        Activated ras Oncogene. Primary peritoneal cells of C57BL/6 mice were immortalized
        by HPV-16 E6 and E7 and then transformed with pEJB expressing activated human c-
        Ha-ras gene. This co-transformation produced a tumorigenic cell line. -

[119]   C57BL/6 mouse peritoneal cells were collected and washed with 1X HBSS. The primary
        single cell suspension was cultured in vitro in RPMI1640, supplemented with 10% fetal
        calf serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium
        pyruvate, and 2 mM nonessential amino acids, and grown at 370C with 5% CO_2.
        Transduction of HPV-16 E6 and E7 genes into primary peritoneal cells was performed
        using the LXSN16E6E7 retroviral vector, kindly provided by Denise A. Galloway (Fred
        Hutchinson Cancer Research Center, Seattle, WA) (Halbert, et al., (1991) J Virol,
        65:473-478). HPV-16 E6- and E7-containing LXSN16E6E7 was used to infect CRIP
        cells to generate recombinant virus with a wide host range. Primary peritoneal cells were
        immortalized by transduction as described previously (Halbert, et al., (1991) J Virol,
        65:473-478). Following transduction, the retroviral supernatant was removed, and cells
        were grown in G418 (0.4 mg/ml) culture medium for an additional 3 days to allow for
        integration and expression of recombinant retroviral genes. The immortalized lung
        (E6+E7+) cells were then transduced with pVEJB expressing activated human c-Ha-ras
        gene, kindly provided by Chi V. Dang (The John Hopkins Hospital, Baltimore, MD), and
        selected with G418 (0.4 mg/ml) and hygromycin (0.2 mg/ml).

Example 6

[120]   Characterization of Histological and Pathological Features of WF-3. 5x104 WF-3 tumor
        cells were injected into C57BL/6 mice intraperitoneally. 4 weeks later, mice were
        sacrificed to examine the formation of ascites and tumors. Removed organs were fixed
        with 4% buffered formaldehyde and histological sections were made, followed by routine
        hematoxylin-eosin staining. Slides were observed under a light microscope.

[121]   Mice were injected with 5x104 WF-3 tumor cells intraperitoneally and sacrificed 4 weeks
        later. This cell line was capable of generating ascites in mice challenged with tumor cells
        intraperitoneally (see Fig. 7A). Morphologically, WF-3 tumor cells showed a papillary
        architecture resembling serous tumors found in the human ovary/peritoneum (Figure 1B).

Furthermore, the tumor showed a high level of mitotic activity, pleiomorphic nuclei, abnormal mitosis, and a high nuclear/cytoplasmic ratio, consistent with a highly malignant neoplasm (see Fig. 7C).

[122] Fig.7 shows the generation and characterization of an ascitogenic ovarian tumor cell line (WF-3). WF-3 tumor cells were injected into C57BL/6 mice intraperitoneally at a dose of 1x105 cells/mouse. Mice were euthanized 4 weeks after tumor challenge (see Fig. 7A) Representative gross picture to demonstrate ascites formation in mice. Note: Mice developed significant ascites with an increase in abdominal girth 4 weeks after tumor challenge. Fig. 7B shows hematoxylin and eosin staining of the explanted tumors viewed at 100x magnification. The tumors displayed a papillary configuration, morphologically consistent with tumors derived from the peritoneum or ovaries. Fig. 7C shows tumors viewed at 400x magnification. The inset displays the features of a WF-3 tumor cell in greater detail.

Example 7

[123] MHC Class I and Class II Presentation of WF-3 Tumor Cells. WF-3 tumor cells were harvested and prepared for flow cytometry analysis. Anti-H-2Kb/H-2Db monoclonal antibody or anti-I-Ab monoclonal antibody was added for the detection of MHC class I and class II expression on WF-3 tumor cells.

[124] WF-3 tumor cells were harvested, trypsinized, washed, and resuspended in FACScan buffer. Anti-H-2Kb/H-2Db monoclonal antibody (Clone 28-8-6, PharMingen, San Diego, CA) or anti-I-Ab monoclonal antibody (Clone 25-9-17, PharMingen, San Diego, CA) was added and incubated for 30 min on ice. After washing twice in FACScan buffer, FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA) was added and incubated for 20 min on ice. Samples were resuspended in FACScan buffer. Analysis was performed on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

[125] Our data indicate that WF-3 is positive for MHC class I expression (Fig. 8A) but negative for MHC class II expression (Fig. 8B). In particular, Fig. 8 shows the MHC class I and II presentation on WF-3 tumor cells. WF-3 tumor cells were harvested, trypsinized, washed, and resuspended in FACSCAN buffer. Anti-H-2Kb/H-2Db monoclonal

antibody or anti-I-Ab monoclonal antibody was added, followed by flow cytometry analysis to detect MHC class I and class II expression on WF-3 tumor cells. Fig. 8A shows WF-3 tumor cells which were positive for MHC class I presentation (thick line) compared to the MHC class I-negative control (thin line). Fig. 8B shows the WF-3 tumor cells which were negative for MHC class II presentation. The thin line indicates staining of the MHC class II-negative control.

Example 8

[126]   Determination of Minimal Tumor Dose of WF-3 Tumor Cells to Lead to Formation of Lethal Ascites. WF-3 tumor cells were injected into C57BL/6 mice intraperitoneally at various doses (1x104, 5x104, 1x105, and 1x106 cells/mouse). Mice were monitored twice a week for formation of ascites and tumors and sacrificed after 90 days. For survival following tumor challenge, mice were challenged intraperitoneally with various doses of WF-3 (1x104, 5x104, 1x105, and 1x106 cells/mouse) and monitored for their survival after tumor challenge.

[127]   As shown in Fig. 9A, all of the mice injected with 5x104, 1x105, and 1x106 cells intraperitoneally formed ascites within 30 days. Meanwhile, 20% of mice injected with 1x104/mouse were tumor-free and without ascites formation 90 days after tumor challenge. All of the mice injected with a dose of 5x104 tumor cells or greater died within 50 days of tumor challenge (Fig. 9B). These data suggest that WF-3 tumor cells are able to lead to formation of ascites and solid tumors in the peritoneum of mice and eventually kill the injected mice at a certain tumor challenge dose.

Example 9

[128]   Mesothelin is Highly Expressed in the WF-3 Preclinical Ovarian Cancer Model. We have performed microarray analysis (Incyte Genomics Corporation, Palo Alto, California) to characterize the gene expression profile of WF-3 compared to pre-WF0. The pre-WF0 cell line was generated by immortalizing mouse primary peritoneal cells with a retroviral vector carrying HPV-16 E6 and E7 genes using a previously described method (Lin, et al., (1996) Cancer Research, 56:21-26). We have chosen pre-WF0 as a reference cell line in order to identify genes in WF-3 that are relevant to tumorigenicity in later stages of ovarian cancer. Table 4 (below) summarizes highly expressed genes present in WF-3 relative to pre-WF0. As shown in Table 4, below, mesothelin is among

the top 10 up-regulated genes in WF-3, suggesting that WF-3 may be a suitable preclinical model for developing mesothelin-specific cancer immunotherapy against ovarian cancer.

Table 4. Summary of Specifically Expressed Genes in WF-3

Marker / Antigen	Sequence Accession #	Balanced differential expression
EGF-containing fibulin-like extracellular matrix protein 1	AI156278	6.5
Mesothelin	AA673869	3.8
alpha-2-HS-glycoprotein	AI386037	3.3
Protein kinase, cGMP-dependent, type II	AA771678	3.2
sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3E	AA241390	3.2
Ankyrin-like repeat protein	AA792499	2.8
RIKEN cDNA 1300019103 gene	AA600596	2.6
Matrix gamma-carboxyglutamate (gla) protein	W88093	2.4
serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium Derived factor). member 1	AA727967	2.3
RIKEN cDNA1200011C15 gene	AA608330	2.2

Example 10

[129] Expression of Mesothelin mRNA and Protein in WF-3 Tumor Cells. We further confirmed the expression of mesothelin by the WF-3 cell line using RT-PCR.

[130] RNA was extracted from WF-3 tumor cells using RNAzol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. RNA concentration was measured and 1 mg of total cellular RNA was reverse transcribed in a 20 ml volume using oligo(dT) as a primer and Superscript reverse transcriptase (Gibco BRL). One ml of cDNA was amplified by the PCR using a set of primers (5'-CCCGAATTCATGGCCTTGCCAACAGCTCGA-3' and 5'-TATGGATCCGCTCAGCCTTAAAGCTGGGAG-3'; SEQ ID NOS: 11 and 12, respectively). The primer was derived from the published murine mesothelin cDNA sequence (Kojima, et al., (1995) J Biol Chem, 270:21984-21990). PCR was performed in a 50 ml reaction mixture with 250 mM of each dNTP, 100 nM of primers, 5 ml of 10 X buffer (New England Biolabs, Berverly, MA), and 1 U of Vent DNA polymerase (New England Biolabs) using 30 cycles (94°C, 1-min denaturation; 55°C, 1-min annealing; and 72°C, 2-min extension). The reaction mixture (10 ml samples) was analyzed using agarose gel electrophoresis (1%) in TAE buffer containing 0.2 mg/ml ethidium bromide.

[131] Murine mesothelin protein shares about 65% similarity with human mesothelin protein. As shown in Fig. 10, we were able to detect mRNA expression of murine mesothelin in WF-3 tumor by RT-PCR (lane 2) but not in the control, B-16 tumor cells (lane 3). Western blot analysis was performed to determine expression of mesothelin protein in WF-3 tumor cells. Tumor cells were stained with anti-mesothelin mouse polyclonal antibodies. Results of the Western blot analysis confirmed that WF-3 was positive for mesothelin protein while B16 melanoma cells were mesothelin-negative (data not shown). Thus, our results indicate that WF-3 cells express mesothelin mRNA and protein.

[132] Fig. 10 shows expression of murine mesothelin in WF-3 tumor cells as demonstrated by RT-PCR with gel electrophoresis. Western blot analysis was also performed to confirm expression (not shown). As shown in Fig. 10, RT-PCR was performed using the Superscript One-Step RT-PCR Kit (Gibco, BRL) and a set of primers: 5'-CCCGAATTCATGGCCTTGCCAA-CAGCTCGA-3' and 5'-TATGGATCCGCTCA GCCTTAAAGCTGGGAG-3' (SEQ ID NOS: 11 and 12, respectively). Western blot

56

analysis was also used to demonstrate the expression of mesothelin protein in WF-3 tumor cells. Tumor cells were stained with anti-mesothelin mouse polyclonal antibody followed by FITC-conjugated goat anti-mouse IgG secondary antibody (data not shown).

Example 11

[133] Mesothelin DNA Cancer Vaccine Immunotherapy. Using the peritoneal tumor model described above we demonstrated the ability of a DNA vaccine encoding mesothelin to generate mesothelin-specific cytotoxic T lymphocyte responses and antitumor effects greater than empty plasmid DNA. These data indicate that a DNA tumor vaccine targeting mesothelin can be used in treating or controlling ovarian carcinomas and other cancers in which mesothelin is highly expressed.

[134] Plasmid DNA Construction. With the availability of the mesothelin-expressing tumor cell line, WF-3, we created DNA vaccines encoding mesothelin to test their antitumor effect against WF-3 in C57BL/6 mice. We used a mammalian cell expression vector, pcDNA3, to generate a DNA vaccine encoding murine full-length mesothelin protein (total length: 625 aa).

[135] For construction of pcDNA3-mesothelin, a DNA fragment encoding mesothelin was first amplified from WF-3 extracted RNA and a set of primers (5'-CCCGAATTCATGGCCT-TGCCAACAGCTCGA-3' and 5'-TATGGATCCGCTCAGCCTTAAAGCTGGGAG-3'; SEQ ID NOS: 11 and 12, respectively) by RT-PCR using the Superscript One-Step RT-PCR Kit (Gibco, BRL) and cloned into the EcoRI/BamHI sites of pcDNA3. The primer was derived from the published murine mesothelin cDNA sequence (11). The accuracy of DNA constructs was confirmed by DNA sequencing.

[136] Vaccination with a DNA Vaccine Encoding Mesothelin Protein Protects Against Challenge with Mesothelin-Expressing Ovarian Tumors. We tested the ability of this pcDNA3-mesothelin DNA vaccine to protect against tumor challenge with WF-3 cells. Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination using a helium-driven gene gun (Bio-rad, Hercules, CA) was performed according to a previously described protocol (Chen, et al., (2000) Cancer Research, 60: 1035-1042. DNA-coated gold particles (1 mg DNA/bullet) were delivered to the shaved

abdominal region of C57BL/6 mice using a helium-driven gene gun (Bio-rad, Hercules, CA) with a discharge pressure of 400 p.s.i.

[137] For the tumor protection experiment, mice (ten per group) were vaccinated intradermally with 2 mg of pcDNA3-mesothelin DNA. One week later, mice received a booster with the same dose. Mice were challenged one week after booster with a lethal injection of $5x104$ WF-3 tumor cells intraperitoneally. Mice were monitored for evidence of ascites formation by palpation and inspection twice a week; the mice were sacrificed at day 90. The percentage of ascites-free mice in each vaccination group was determined.

[138] Our data indicated that pcDNA3-mesothelin generated a high degree of protection (60%) against WF-3 tumor challenge. Controls were vaccinated with pcDNA3 vector alone (0%) or were not vaccinated (0%). Fig. 11 shows *in vivo* tumor protection experiments against WF-3 tumor growth using mesothelin-specific DNA vaccines.

Example 12

[139] Vaccination with pcDNA3-mesothelin Generate Mesothelin-Specific Cytotoxic Immune Responses. CD8+ T lymphocytes are important effector cells for mediating antitumor immunity. Cytotoxic T lymphocyte (CTL) assays were performed to determine the cytotoxic effect of mesothelin-specific CD8+ T cells generated by the pcDNA3-mesothelin DNA vaccine. Splenocytes from vaccinated mice served as effector cells after being cultured with cell lysates containing mesothelin protein. WF-3 tumor cells served as target cells.

[140] Generation of Mesothelin-Containing Cell Lysates from Transfected 293 Db,Kb Cells. To generate mesothelin containing cell lysates to pulse splenocytes for the CTL assays, a total of 20 mg of pcDNA3-mesothelin or empty plasmid DNA was transfected into $5x10^6$ Db,Kb cells with lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. The transfected 293 Db,Kb cells were collected 40–44 h after transfection, then treated with three cycles of freeze-thaw. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to vendor's protocol. Cell lysates containing mesothelin were used to pulse splenocytes obtained from the various vaccinated mice as described below.

[141] Cytotoxic T Lymphocyte (CTL) Assays. Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) using CytoTox96 non-radioactive cytotoxicity assay kits (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, splenocytes were harvested from vaccinated mice (5 per group) and pooled 1 week after the last vaccination. Splenocytes were pulsed with 20 mg of cell lysates in a total volume of 2 ml of RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2mM nonessential amino acids in a 24-well tissue culture plate for 6 days as effector cells. WF-3 tumor cells were used as target cells. WF-3 cells were mixed with splenocytes at various effector/target (E:T) ratios. After 5 hr incubation at 370C, 50µl of the cultured media were collected to assess the amount of LDH in the cultured media according to the manufacturer's protocol. The percentage of lysis was calculated from the following equation: 100 X (A-B)/(C-D), where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value.

[142] Statistical Analysis. Statistical determinations were made using the Student's t-test. Two-sided P values are presented in all experiments, and significance was defined as P < 0.05. No mice were excluded from statistical evaluations.

[143] As shown in Fig. 12, vaccination with pcDNA3-mesothelin generated a significant percentage of specific lysis compared to vaccination with pcDNA3 or no vaccination (P<0.001, one-way ANOVA). These results indicate that vaccination with pcDNA3-mesothelin DNA is capable of generating mesothelin-specific T cell-mediated specific lysis of WF-3.

[144] Cytotoxic T Lymphocyte (CTL) assays which demonstrate specific lysis induced by vaccination with mesothelin-specific DNA vaccines. Mice (5 per group) were immunized with various DNA vaccines intradermally. Mice received a booster with the same dose one week later. Splenocytes from mice were pooled 14 days after vaccination. To perform the cytotoxicity assay, splenocytes were cultured with mesothelin protein for 6 days and used as effector cells. WF-3 tumor cells served as target cells. WF-3 cells were mixed with splenocytes at various E:T ratios. Cytolysis was determined by quantitative measurements of LDH. Note: The pcDNA3-mesothelin DNA vaccine

generated a significantly higher percentage of specific lysis than the other DNA vaccines (P<0.001). The data presented in this figure are from one representative experiment of two performed.

Example 13

[145]   In this example, we utilize an attenuated strain of *Salmonella typhimurium* as a vehicle for oral genetic immunization. PcDNA3.1/myc-His(-) vectors expressing a myc-tagged version of mesothelin were constructed. Following immunization with the recombinant *S. typhimurium* aroA strain harboring the mesothelin expression vector, we are able to detect high levels of expression of the mesothelin/myc fusion protein using an anti-myc antibody by immunoassay. The *S. typhimurium* auxotrophic aroA strain SL7202 *S. typhimurium* 2337-65 derivative *hisG46*, DEL407 [*aroA::*Tn10(Tc-s)]), is used as carrier for these *in vivo* studies (see Darji et al. (1997) Cell 91: 761-775; Darji et al. (2000) FEMS Immunology and Medical Microbiology 27: 341-9). This *S. typhimurium*-based mesothelin DNA vaccine delivery system is then used to test whether this vaccine can protect ovarian cancer cells challenge using our WF-3 tumor model system.

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We claim:

1. A method of inducing a T-cell response to a tumor that overexpresses mesothelin relative to normal tissue from which the tumor is derived, said method comprising:

    administering to a patient who has said tumor or who has had said tumor removed, a vaccine comprising a polypeptide comprising an MHC Class I-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I which is expressed by the patient, whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

2. The method of claim 1 wherein the tumor is selected from the group consisting of ovarian cancer, pancreatic cancer, mesothelioma, and squamous cell carcinoma.

3. The method of claim 1 wherein the tumor is a pancreatic cancer.

4. The method of claim 1 wherein the tumor is an ovarian cancer.

5. The method of claim 1 wherein epitope is selected from the group consisting of: SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

6. The method of claim 1 wherein the polypeptide is mature mesothelin.

7. The method of claim 1 wherein the polypeptide is the primary translation product of mesothelin.

8. The method of claim 1 wherein a mixture of said polypeptides is administered.

9. The method of claim 8 wherein said polypeptides bind to a plurality of allelic forms of MHC Class I molecules.

10. The method of claim 8 wherein said polypeptides bind to a single allelic form of MHC Class I molecules.

11. The method of claim 1 wherein the polypeptide is selected as being an MHC class I-binding epitope using an algorithm.

12. The method of claim 1 wherein the polypeptide is selected as being an MHC class I-binding epitope using two algorithms.

13. The method of claim 1 wherein the T-cell response is induction of specific CD8^{+} T cells.

14. The method of claim 1 wherein the vaccine is acellular.

15. The method of claim 1 wherein the vaccine comprises a bacterium selected from the group consisting of: *Shigella flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella typhimurium, Salmonella typhi,* and mycobacterium.

16. The method of claim 1 wherein the vaccine is administered in sufficient amount to induce tumor regression.

17. The method of claim 1 wherein the vaccine is administered in sufficient amount to keep the patient tumor-free after removal of the tumor.

18. A method of inducing a T-cell response to a tumor that overexpresses mesothelin relative to normal tissue from which the tumor is derived, said method comprising:

> administering to a patient who has said tumor or who has had said tumor removed, a vaccine comprising a polypeptide comprising an MHC Class II-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class II which is expressed by the patient, whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

19. A method of inducing a T-cell response to tumor cells that overexpress mesothelin relative to normal cells from which the tumor cells are derived, said method comprising:

> administering to a patient who is at risk of developing a tumor that overexrpresses mesothelin a vaccine comprising a polypeptide comprising an MHC class I-binding epitope of mesothelin or an MHC class II-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I or class II which is expressed by the patient, whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

20. The method of claim 19 wherein the patient has been exposed to a carcinogen which is known to induce tumors which overexpress mesothelin relative to normal tissue from which the tumor is derived.

21. The method of claim 20 wherein the carcinogen is asbestos.

22. A method of inducing a T-cell response to a tumor which overexpresses mesothelin relative to normal tissue from which it is derived, said method comprising:

> administering to a patient who has said tumor or who has had said tumor removed, a vaccine comprising a polynucleotide encoding a polypeptide comprising an MHC Class I-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I which is expressed by the patient, whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

23. The method of claim 22 wherein the tumor is selected from the group consisting of ovarian cancer, pancreatic cancer, mesothelioma, and squamous cell carcinoma.

24. The method of claim 22 wherein the tumor is a pancreatic cancer.

25. The method of claim 22 wherein the tumor is an ovarian cancer.

26. The method of claim 22 wherein epitope is selected from the group consisting of:
    SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID
    NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and
    LYPKARLAF (SEQ ID NO: 6).

27. The method of claim 22 wherein the polypeptide is mature mesothelin.

28. The method of claim 22 wherein the polypeptide is primary translation product of
    mesothelin.

29. The method of claim 22 wherein the vaccine comprises one or more polynucleotides
    encoding a mixture of said polypeptides.

30. The method of claim 29 wherein said polypeptides bind to a plurality of allelic forms of
    MHC Class I molecules.

31. The method of claim 29 wherein said polypeptides bind to a single allelic form of MHC
    Class I molecules.

32. The method of claim 22 wherein the polypeptide is selected as being an MHC class I-
    binding epitope using an algorithm.

33. The method of claim 22 wherein the polypeptide is selected as being an MHC class I-
    binding epitope using two algorithms.

34. The method of claim 22 wherein the T-cell response is induction of specific $CD8^+$ T
    cells.

35. The method of claim 22 wherein the vaccine is acellular.

36. The method of claim 22 wherein the vaccine comprises a bacterium selected from the
    group consisting of: *Shigella flexneri, E. coli, Listeria monocytogenes, Yersinia
    enterocolitica, Salmonella typhimurium, Salmonella typhi,* and mycobacterium.

37. The method of claim 22 wherein the vaccine is administered in sufficient amount to
    induce tumor regression.

38. The method of claim 22 wherein the vaccine is administered in sufficient amount to keep
    the patient tumor-free after removal of the tumor.

39. A method of inducing a T-cell response to a tumor that overexpresses mesothelin relative
    to normal tissue from which the tumor is derived, said method comprising:
    administering to a patient who has said tumor or who has had said tumor
    removed, a vaccine comprising a polynucleotide encoding a polypeptide
    comprising an MHC Class II-binding epitope of mesothelin, wherein the epitope
    binds to an allelic form of MHC class II which is expressed by the patient,

whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

40. A method of inducing a T-cell response to tumor cells that overexpress mesothelin relative to normal cells from which the tumor cells are derived, said method comprising: administering to a patient who is at risk of developing a tumor that overexrpresses mesothelin a vaccine comprising a polynucleotide encoding a polypeptide comprising an MHC class I-binding epitope of mesothelin or an MHC class II- binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I or class II which is expressed by the patient, whereby a T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells.

41. The method of claim 40 wherein the patient has been exposed to a carcinogen which is known to induce tumors which overexpress mesothelin relative to normal tissue from which the tumor is derived.

42. The method of claim 41 wherein the carcinogen is asbestos.

43. A method of identifying immunogens useful as candidates for anti-tumor vaccines, comprising:
selecting a protein which is expressed by a tumor and which is minimally or not expressed by normal tissue from which the tumor is derived;
testing lymphocytes of humans who have been vaccinated with a vaccine which comprises said protein to determine if said lymphocytes comprise CD8+ T cells or CD4+ T cells which are specific for said protein, wherein the presence of said CD8+ T cells or CD4+ T cells indicates that the protein is a candidate for use as an anti-tumor vaccine.

44. The method of claim 43 wherein said humans have exhibited an anti-tumor immune response.

45. The method of claim 43 wherein the vaccine comprises whole tumor cells.

46. The method of claim 44 wherein the anti-tumor immune response results in prolonged disease-free survival post-surgical tumor removal relative to a similar population which has not been vaccinated.

47. The method of claim 44 wherein the anti-tumor immune response results in tumor regression.

48. The method of claim 44 wherein the anti-tumor immune response results in prolonged survival time.

49. The method of claim 44 wherein the anti-tumor immune response is delayed type hypersensitivity to autologous tumor cells.

50. The method of claim 43 wherein said lymphocytes are also tested to determine if they comprise CD8+ T cells or CD4+ T cells specific for an antigen not expressed by the vaccine.

51. The method of claim 43 wherein the humans are divided into two groups based on their response to the vaccine, wherein a first group comprises responders and a second group comprises non-responders, wherein if said CD8+ T cells or CD4+ T cells are found more frequently in responders than in non-responders then the protein is identified as more likely to be useful in an anti-tumor vaccine.

52. The method of claim 51 wherein responders display a DTH response to autologous tumor cells but non-responders do not display the response.

53. The method of claim 51 wherein responders have a longer period of disease free survival than non-responders.

54. A method of predicting future response to a tumor vaccine comprising at least one T-cell epitope of mesothelin in a patient who has received the vaccine, comprising:

   testing lymphocytes of the patient to determine if the lymphocytes comprise CD8+ T cells or CD4+ T cells which are specific for mesothelin, wherein the presence of said CD8+ T cells or CD4+ T cells predicts a longer survival time than the absence of said $CD8^+$ T cells.

55. The method of claim 54 wherein the vaccine comprises whole tumor cells.

56. The method of claim 54 wherein the vaccine comprises pancreatic tumor cells and the antigen is mesothelin.

57. The method of claim 54 wherein the vaccine comprises ovarian tumor cells and the antigen is mesothelin.

58. The method of claim 54 wherein the vaccine comprises mesothelioma cells and the antigen is mesothelin.

59. A vaccine which induces a $CD8^+$ T cell or $CD4^+$ T cell response, comprising:

   a polypeptide comprising an MHC Class I- or Class II-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I of class II which is expressed by the patient, whereby a $CD8^+$ T cell or $CD4^+$ T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells; and

   a carrier for stimulating a $CD8^+$ T cell or $CD4^+$ T cell immune response.

67

60. The vaccine of claim 59 wherein the polypeptide comprises an MHC Class I-binding epitope.

61. The vaccine of claim 59 wherein the polypeptide comprises between 6 and 20 amino acid residues.

62. The vaccine of claim 59 wherein the polypeptide comprises an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

63. The vaccine of claim 59 wherein the carrier is CD40/CD40 ligand.

64. The vaccine of claim 59 wherein the carrier is OX-40/OX-40 ligand.

65. The vaccine of claim 59 wherein the carrier is a CTLA-4 antagonist.

66. The vaccine of claim 59 wherein the carrier is GM-CSF.

67. A vaccine which induces a CD8$^+$ T cell or CD4$^+$ T cell response, comprising:

   a polynucleotide encoding a polypeptide comprising an MHC Class I- or Class II-binding epitope of mesothelin, wherein the epitope binds to an allelic form of MHC class I or Class II which is expressed by the patient, whereby a CD8$^+$ T cell or CD4$^+$ T-cell response to mesothelin is induced, wherein the vaccine does not comprise whole tumor cells; and

   a carrier for stimulating a CD8$^+$ T cell or CD4$^+$ T cell immune response.

68. The vaccine of claim 67 wherein the carrier is CD40/CD40 ligand.

69. The vaccine of claim 67 wherein the carrier is OX-40/OX-40 ligand.

70. The vaccine of claim 67 wherein the carrier is a CTLA-4 antagonist.

71. The vaccine of claim 67 wherein the carrier is GM-CSF.

72. The vaccine of claim 67 wherein the polypeptide comprises an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

73. The vaccine of claim 59 which comprises a bacterium.

74. The vaccine of claim 67 which comprises a bacterium.

75. The vaccine of claim 73 wherein the bacterium is selected from the group consisting of : *Shigella flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella typhimurium, Salmonella typhi,* and mycobacterium.

76. The vaccine of claim 74 wherein the bacterium is selected from the group consisting of :
    *Shigella flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella
    typhimurium, Salmonella typhi,* and mycobacterium.

77. An isolated polypeptide of 9 to 25 amino acid residues comprising an epitope selected
    from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO:
    2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL
    (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

78. A fusion protein comprising a first and a second portion, wherein the first portion
    comprises a polypeptide of 9 to 25 amino acid residues comprising an epitope selected
    from the group consisting of SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO:
    2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL
    (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6), and the second portion comprises
    a segment of at least 6 amino acid residues, wherein the sequence of said second portion
    is not in mesothelin.

79. An expression vector which encodes a polypeptide of 9 to 25 amino acid residues
    comprising an epitope selected from the group consisting of SLLFLLFSL (SEQ ID NO:
    1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3); ALQGGGPPY
    (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF (SEQ ID NO: 6).

80. A bacterium which comprises the expression vector of claim 79.

81. The bacterium of claim 80 which is selected from the group consisting of *Shigella
    flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella
    typhimurium, Salmonella typhi,* and mycobacterium.

82. An expression vector which encodes the fusion protein of claim 78.

83. A bacterium which comprises the expression vector of claim 82.

84. The bacterium of claim 83 which is selected from the group consisting of *Shigella
    flexneri, E. coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella
    typhimurium, Salmonella typhi,* and mycobacterium.

85. An isolated antibody that binds to an epitope selected from the group consisting of
    SLLFLLFSL (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID
    · NO: 3); ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and
    LYPKARLAF (SEQ ID NO: 6).

86. A T-cell line that binds to an epitope selected from the group consisting of SLLFLLFSL
    (SEQ ID NO: 1); VLPLTVAEV (SEQ ID NO: 2); ELAVALAQK (SEQ ID NO: 3);

ALQGGGPPY (SEQ ID NO: 4); FYPGYLCSL (SEQ ID NO: 5); and LYPKARLAF
(SEQ ID NO: 6).

87. The polypeptide of claim 77 which is bound to an MHC Class I molecule.

88. The fusion protein of claim 78 which is bound to an MHC Class I molecule.

89. The vaccine of claim 59 wherein the carrier is an MHC Class I molecule.

90. The polypeptide of claim 87 wherein the MHC Class I molecule is on a dendritic cell.

91. The fusion protein of claim 88 wherein the MHC Class I molecule is on a dendritic cell.

92. The vaccine of claim 89 wherein the MHC Class I molecule is on a dendritic cell.

93. The polypeptide of claim 87 wherein the MHC Class I molecule is on an antigen
presenting cell.

94. The polypeptide of claim 88 wherein the MHC Class I molecule is on an antigen
presenting cell.

95. The vaccine of claim 89 wherein the MHC Class I molecule is on an antigen presenting
cell.

96. A method of predicting future response to a tumor vaccine in a patient who has received
the vaccine, comprising:

testing the patient to determine if the patient has a delayed type hypersensitivity
(DTH) response to mesothelin, wherein the presence of said response predicts a
longer survival time than the absence of said response.

97. The method of claim 96 wherein the vaccine comprises whole tumor cells.

98. The method of claim 96 wherein the vaccine comprises pancreatic tumor cells.

99. The method of claim 96 wherein the vaccine comprises ovarian tumor cells.

100.   The method of claim 96 wherein the vaccine comprises mesothelioma cells.

101.   A recombinant mouse cell line which comprises peritoneal cells which have been
transformed by HPV-16 E6 and E7 and an activated oncogene wherein the cell line is
capable of forming ascites and tumors upon intraperitoneal injection into an
immunocompetent mouse.

102.   The recombinant mouse cell line of claim 101 wherein the activated oncogene is
an activated c-Ha-ras.

103.   The recombinant mouse cell line of claim 101 which expresses mesothelin.

104.   The recombinant mouse cell line of claim 101 which is WF-3.

105.   A mouse model comprising:
a mouse which has been injected with the recombinant mouse cell line of claim
101.

106.   The mouse model of claim 105 which is immunocompetent.

107.   A method of testing a substance to determine if it is a potential drug for treating a
cancer selected from the group consisting of ovarian cancer, pancreatic cancer,
mesothelioma, and squamous cell carcinoma, comprising:

> contacting the mouse model of claim 105 with a test substance; and
>
> determining if the test substance causes regression of a tumor in the mouse model,
> diminution of ascites volume in the mouse model, or longer survival time in the
> mouse model.

108.   A method of testing a substance to determine if it is a potential drug for treating a
cancer selected from the group consisting of ovarian cancer, pancreatic cancer,
mesothelioma, and squamous cell carcinoma, comprising:

> contacting a mouse with a test substance;
>
> injecting the mouse with the recombinant cell line of claim 101, and
>
> determining if the test substance causes regression of a tumor in the mouse,
> diminution of ascites volume in the mouse, or longer survival time in the mouse.

109.   The vaccine of claim 59, wherein the polypeptide is mesothelin.

110.   The method of claim 1, wherein the polypeptide is mesothelin.

111.   The method of claim 22, wherein the polypeptide is mesothelin.

112.   The vaccine of claim 67, wherein the polypeptide is mesothelin.

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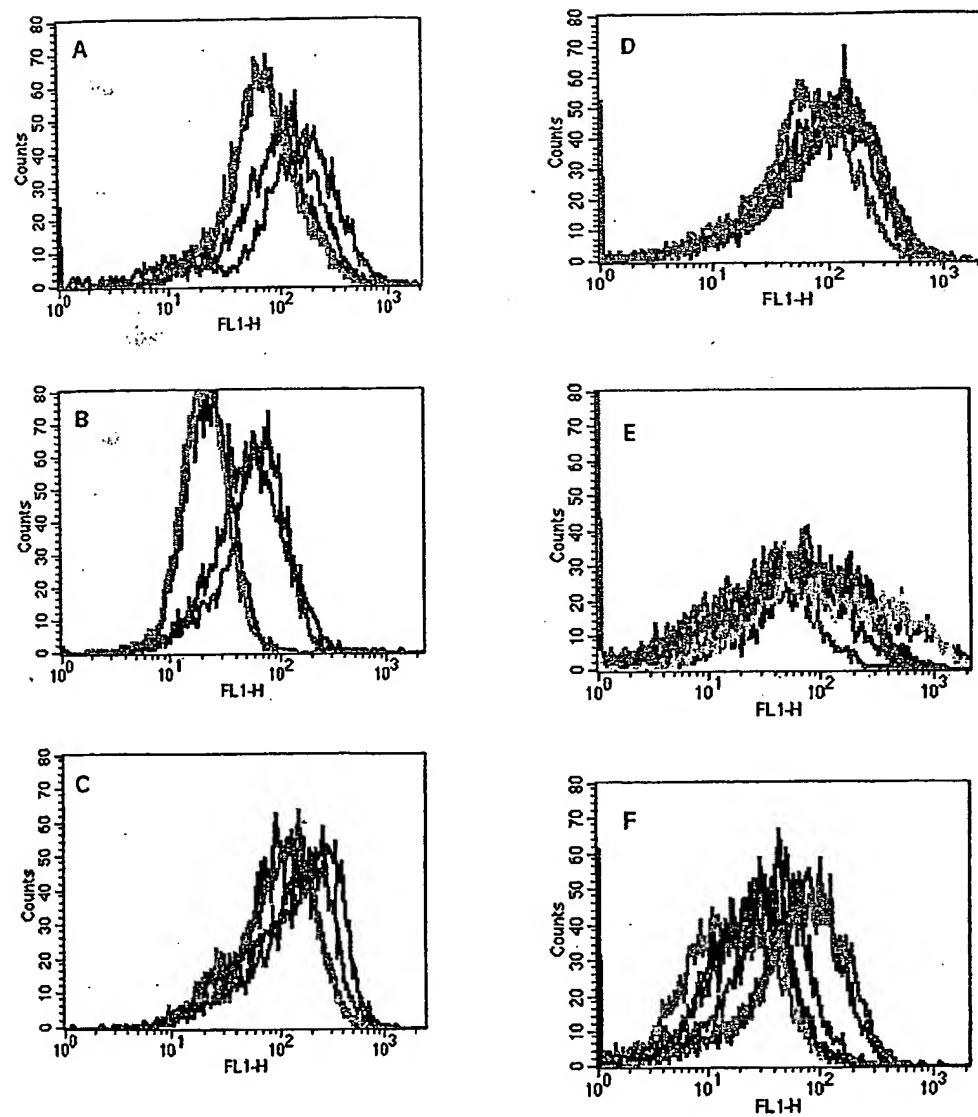
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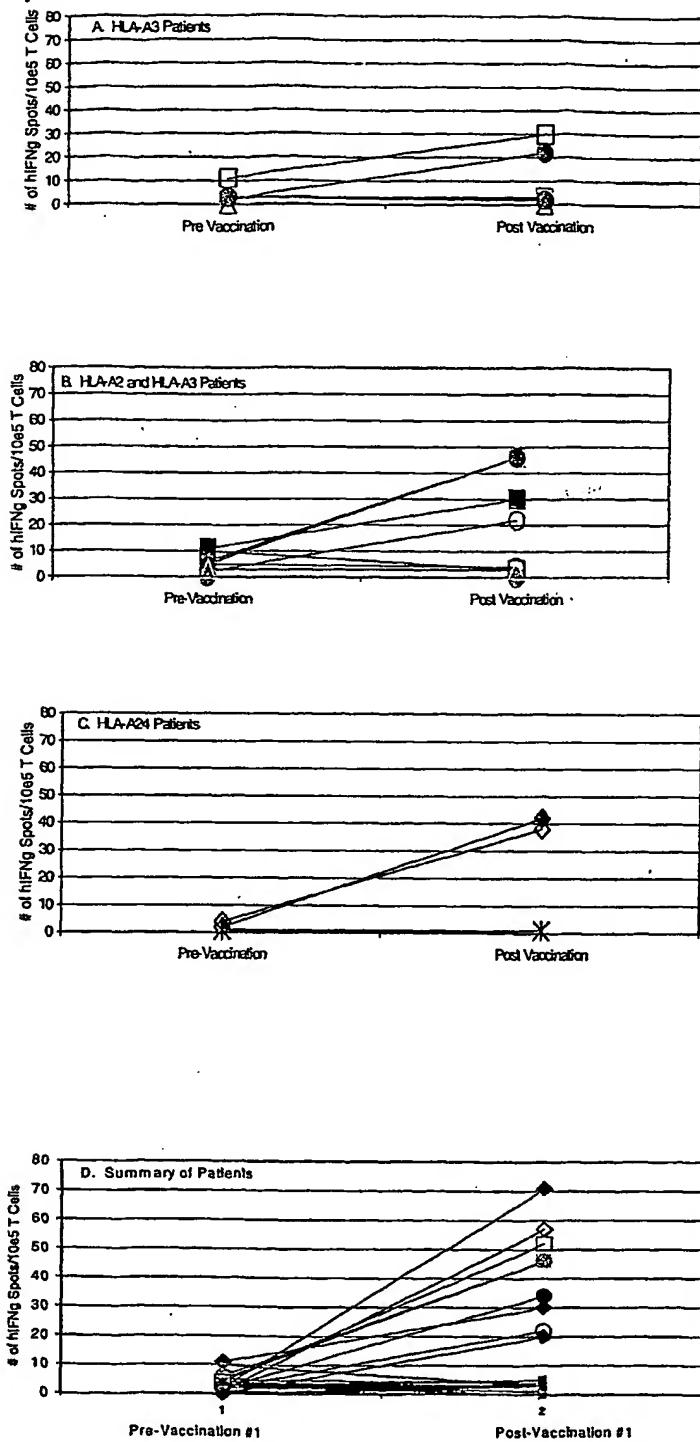
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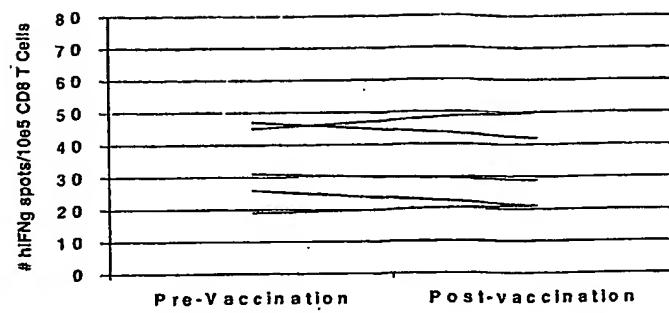
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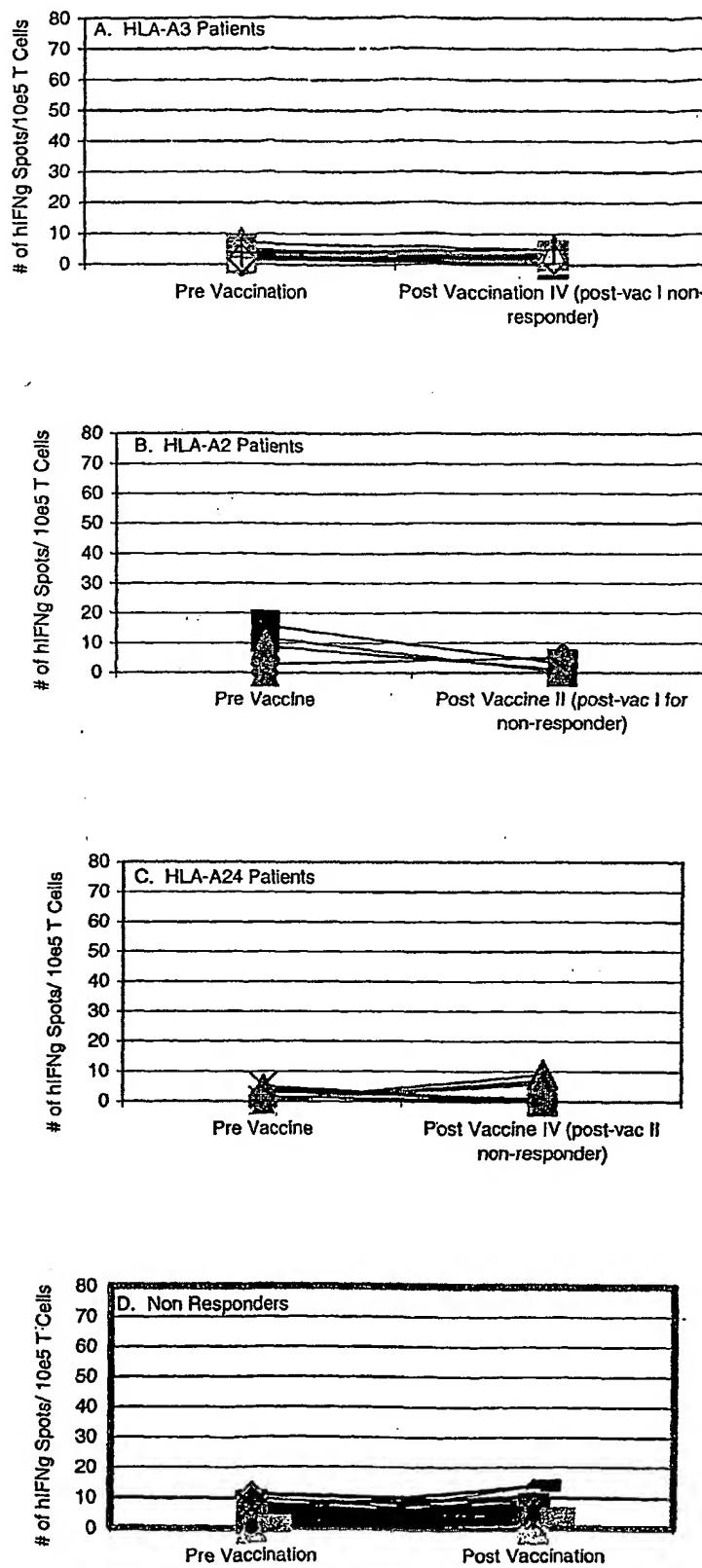
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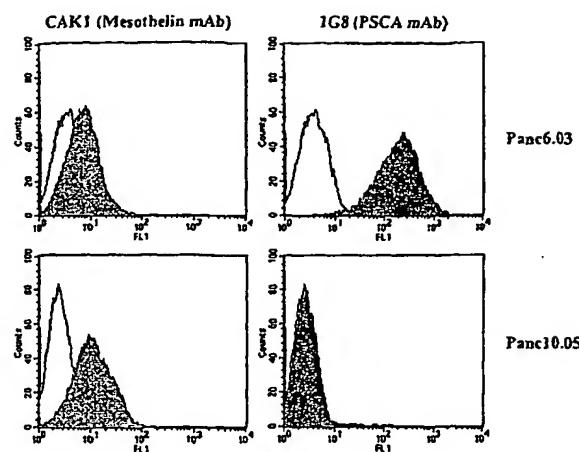
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Panc6.03

Panc10.05

Expression of Mesothelin and PSCA on Panc6.03 and Panc10.05

Figure 6

A. HLA-A3 Patients


B. HLA-A2 and HLA-A3 Patients

C. HLA-A24 Patients
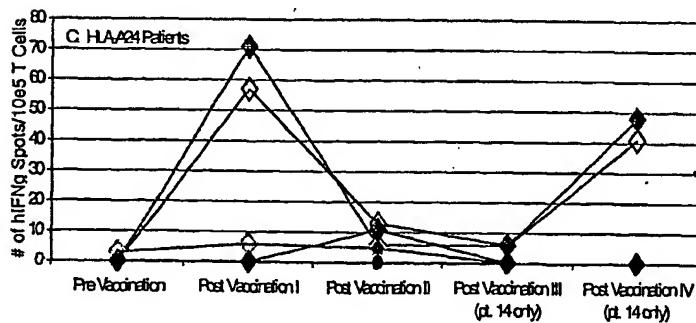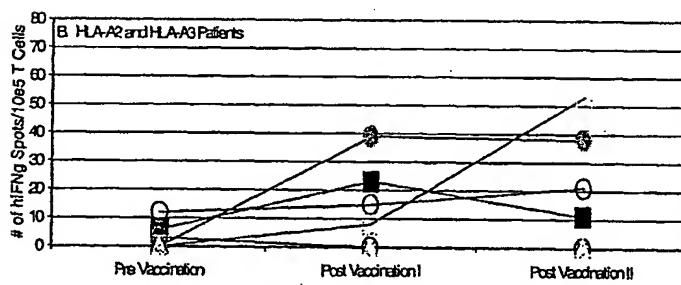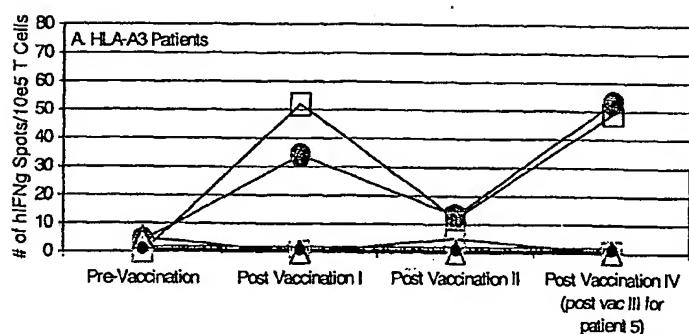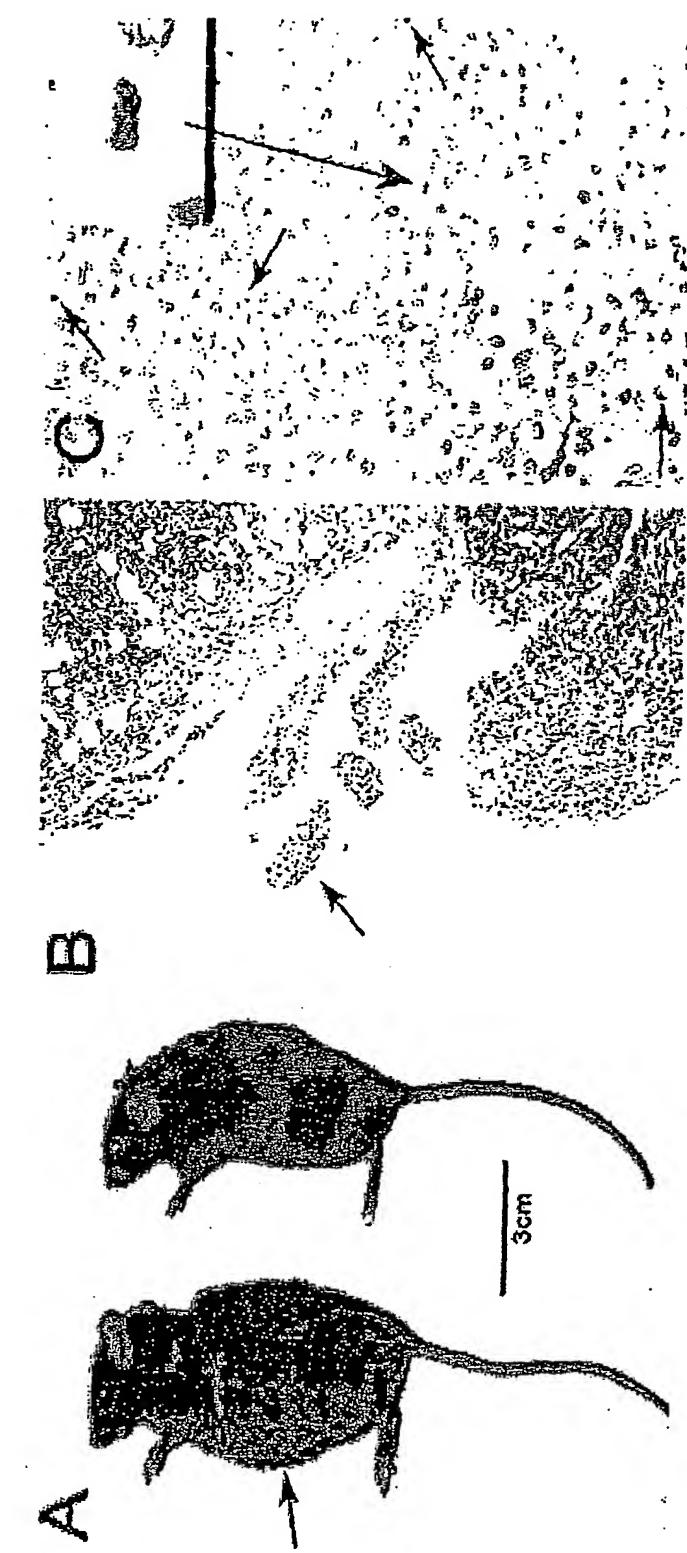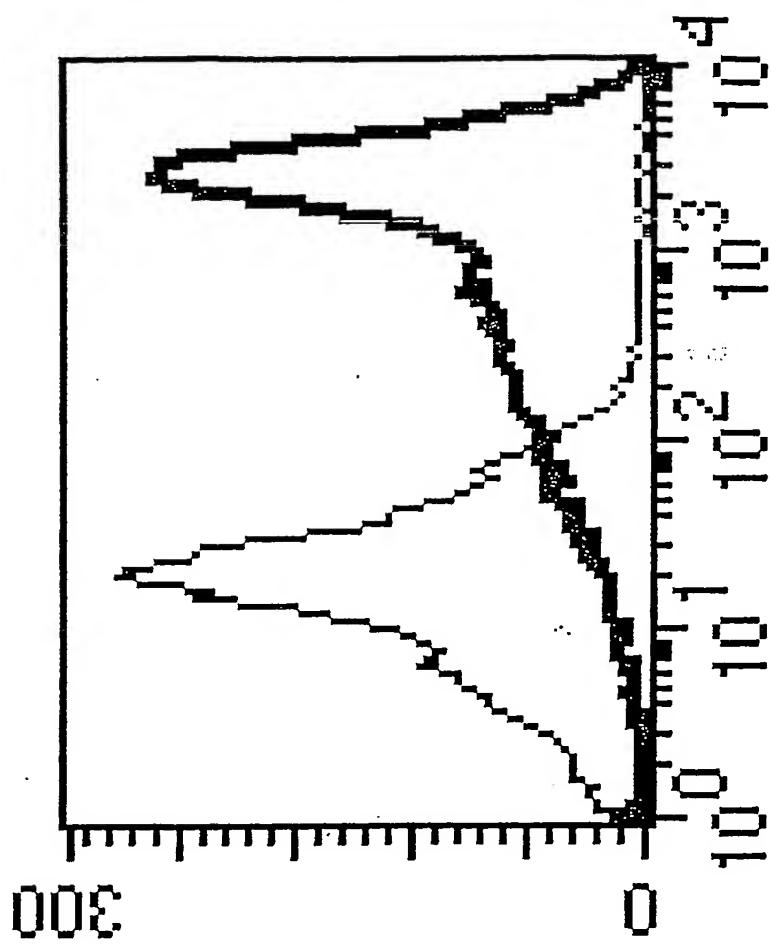
FIGURE 7

FIGURE 8A

FIGURE 8B

FIGURE 9A

FIGURE 9B

% of Surviving Mice

Days After Tumor Challenge

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1x10^4
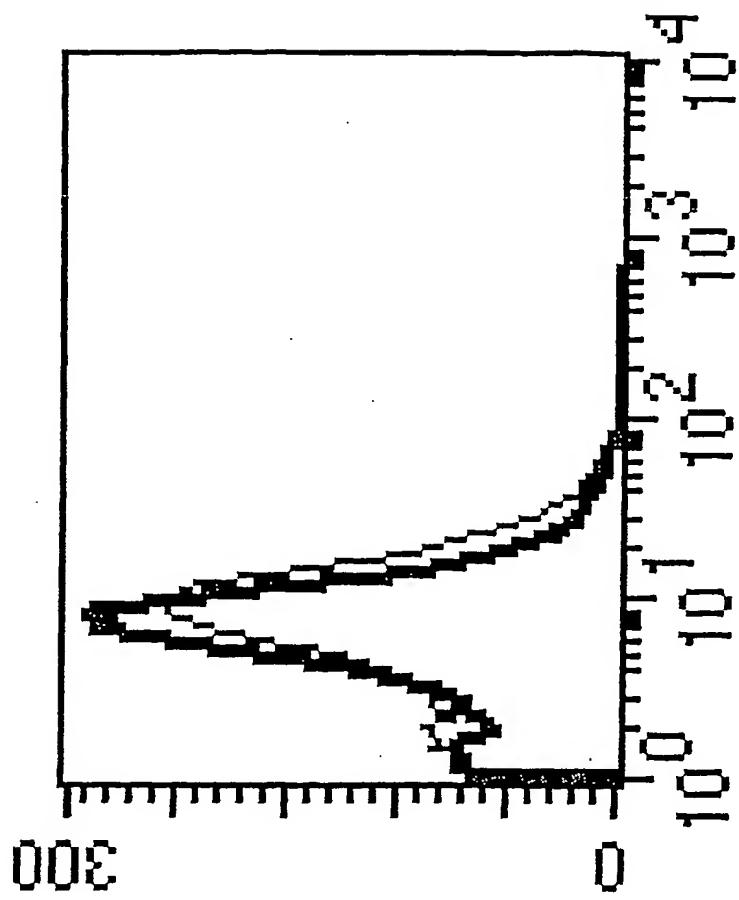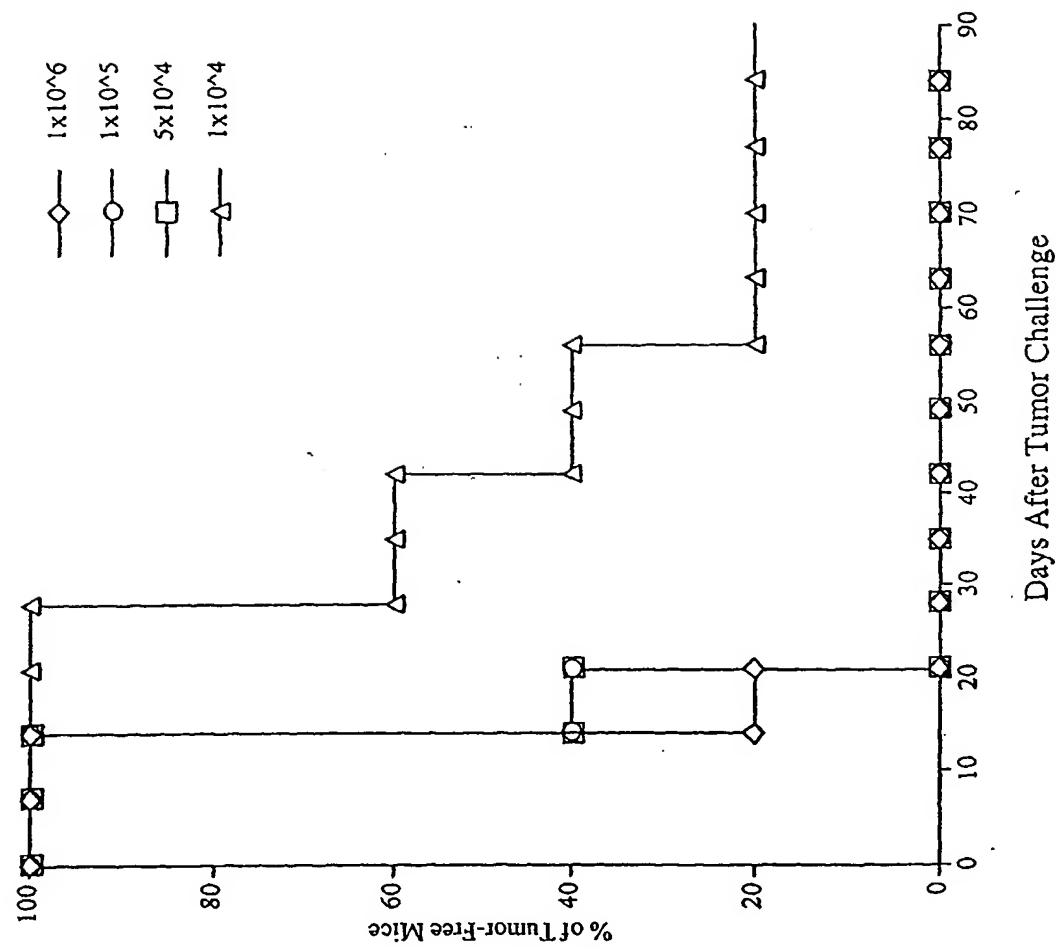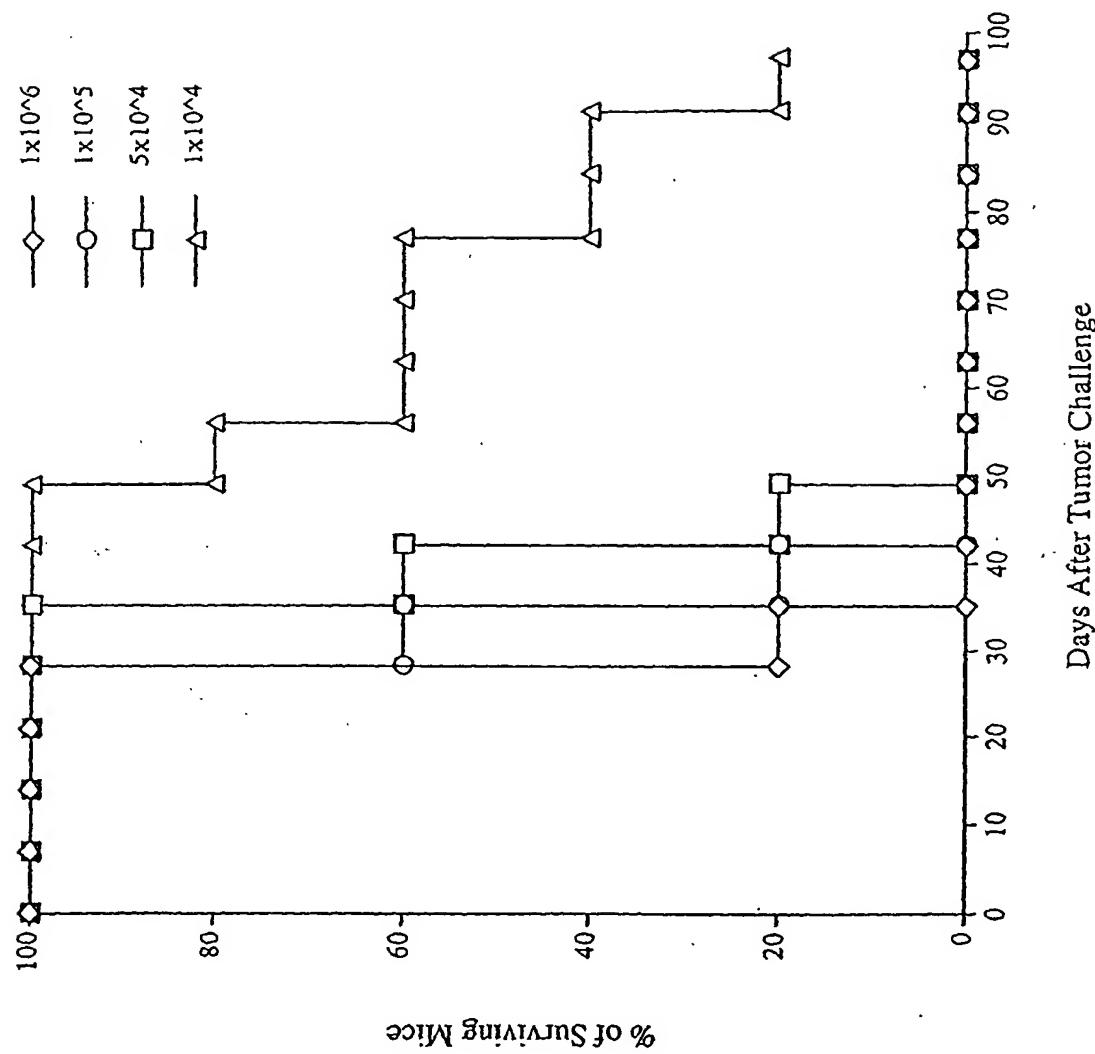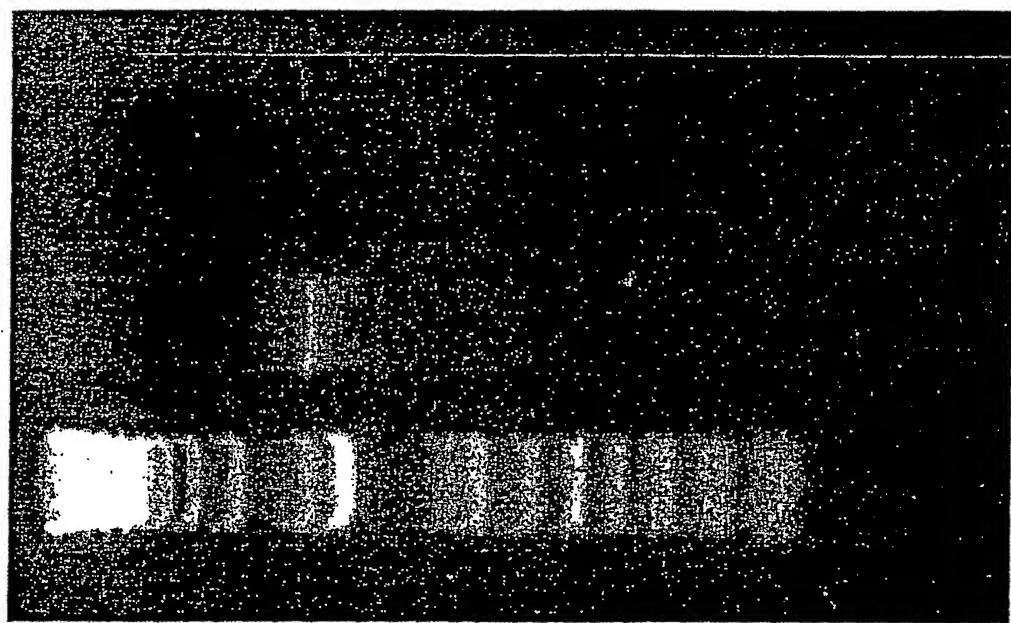
Figure 10

mesothelin
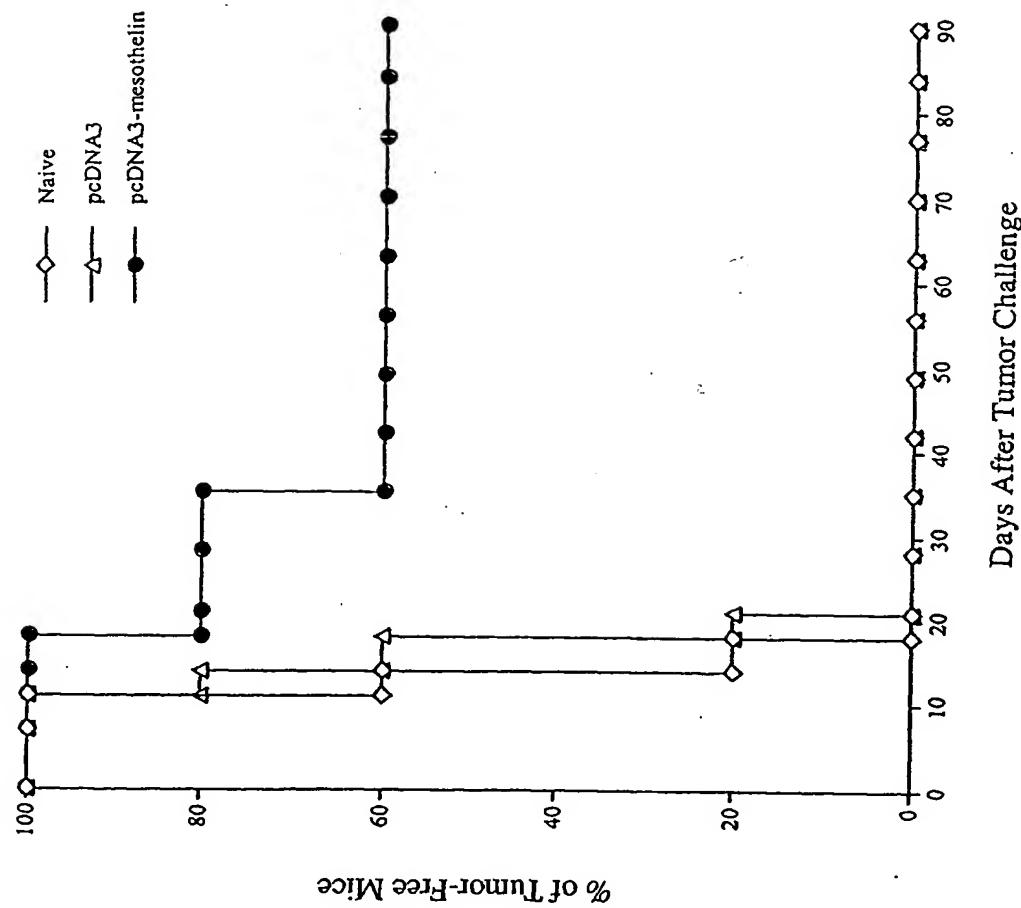
FIGURE 11

FIGURE 12
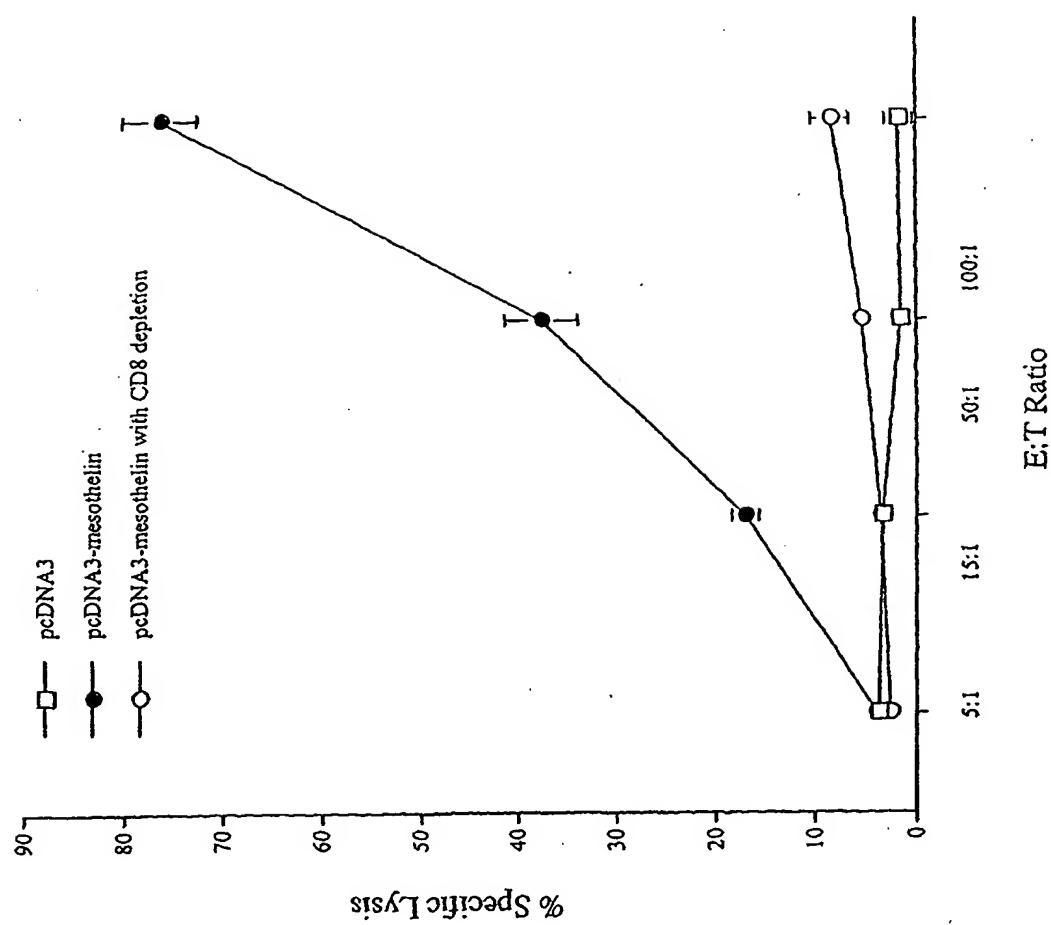
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